University of Alberta

Plant amino acid metabolism under hypoxia and carbon starvation

by

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Abstract

Efficient assimilation of inorganic nitrogen into amino acids is fundamental to plant growth and development. Therefore, biosynthesis of amino acids in plants has been the subject of intensive research. Nevertheless, the complex nature of plant amino acid metabolism is not fully understood. The current study analyzed the enzymes involved in the metabolism of alanine, GABA and glutamate since uncertainty remains as to the physiological roles of these enzymes and metabolites. To characterize the *in vivo* functions of the corresponding enzymes, a reverse genetics approach was taken using *Arabidopsis thaliana*.

Regarding alanine and GABA, their increased syntheses under low oxygen stress were examined since this adaptive response undergoes a dramatic change in the metabolism of these amino acids. Firstly, alanine aminotransferase (AlaAT) was studied since this enzyme had been thought to be responsible for the accumulation of alanine under hypoxia. However, this was not supported in the experiment as the mutants defective in *AlaAT* accumulated alanine under hypoxia. Instead, the slowed decline in alanine during the post-hypoxic periods was observed in the *alaat* mutants, suggesting that the physiological role of AlaAT lies in the breakdown of alanine. Secondly, the involvement of the GABA-shunt in the hypoxic alanine accumulation was investigated since this pathway produces alanine along with the conversion of GABA to its downstream metabolites. The analyses of the mutants for the enzymes catalyzing the first and second steps of the GABA-shunt, glutamate decarboxylase (GAD) and GABA-transaminase (GABA-T), respectively, demonstrated the contribution of this pathway to the hypoxic alanine accumulation in roots.

Glutamate dehydrogenase (GDH), a key player for the metabolism of glutamate, was also studied. Prior to this research, the *in vivo* role of this enzyme has been ambiguous for several decades. The physiological role of GDH was characterized here by analyzing a double mutant defective in the two known *GDH* genes. The increased susceptibility of the double mutant to prolonged darkness demonstrated the involvement of GDH in funneling glutamate carbon to the respiratory pathway. Furthermore, monitoring changes in the amino acid contents during dark treatments suggested the central role of GDH in the catabolism of many amino acids.

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List of Abbreviations

ABA	Abscisic acid
ADP	Adenine diphosphate
ATP	Adenine triphosphate
bp	Base pair
BSA	Bovine serum albumin
C	Carbon
°C	Degree Celsius
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
Fd	Ferredoxin
g; mg; µg	Gram; milligram; microgram
GUS	β-Glucuronidase
h	Hours
HPLC	High performance liquid chromatography
Kb	Kilo base
KD	Kilo dalton
L; ml; µl	Liter; milliliter; microliter
M; mM; µM	Molar; millimolar; micromolar
Min	Minutes
MMt	Million metric tons
mRNA	messenger RNA
MS	Murashige and Skoog
MSX	Methionine sulfoximine
N	Nitrogen
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate

NMR	Nuclear magnetic resonance (spectrometry)
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulphonylfluoride
PVPP	Polyvinylpolypyrrolidone
QTL	Quantitative trait loci
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase-PCR
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE	Standard error
TILLING	Targeting induced local lesions in genomes
TCA	Tricarboxylic acid
Tris	Tris (hydroxymethyl) aminomethane
U	Units
UV	Ultraviolet
v/v; v/w; w/v	volume per volume; volume per weight; weight per volume
vol	volume
WT	Wild-type

1. General Introduction

1.1. Nitrogen as a plant nutrient

Nitrogen (N) is an essential component of many organic compounds such as amino acids and nucleotides. The nitrogen moieties of these organic compounds found in plants are all derived from the assimilation of inorganic N acquired in roots. In both natural and agricultural settings, the demand of plants for this element is generally the highest among the essential macronutrients required for plant growth and development. Nonetheless, the supply of biologically active N seldom meets the demand of plants in the modern agricultural systems. The anthropogenic input of fixed N is thus indispensable in the modern agriculture practices to obtain the optimal crop yield. Therefore, the biogeochemical N cycle and the current issues pertinent to the agricultural N fertilizer use are discussed first to begin this thesis.

1.1.1. Nitrogen fixation

Among the many forms of N present in the biosphere, nitrate (NO_3^-) and ammonium (NH_4^+) are the principal sources of N available to plants. The gaseous form of nitrogen (N_2) exists in vast quantity in the air, accounting for nearly 80% of the atmospheric gas by volume. However, plants cannot use this abundant form of N due to the high energy required to break the stable triple covalent bond of this inert molecule. Nitrogen fixation, a reaction that produces nitrate or ammonium from gaseous N_2, occurs in both natural and industrial processes (summarized in Table 1.1; Taiz and Zeiger, 1998). In nature, lightning is the major process resulting in non-biological N fixation. Lightning triggers the generation of reactive oxygen species in the atmosphere that attack molecular N_2 to form nitric acid (HNO_3) (Taiz and Zeiger, 1998). A small amount of nitric acid is also produced through a photochemical reaction between nitric oxide and ozone (Taiz and Zeiger, 1998). The nitric acid formed in this manner is then provided to the terrestrial ecosystem with rainfall. Nevertheless, the amount of fixed N which occurs as a result of these processes accounts for only about 10% of the N fixation by natural processes with the remaining portion resulting from the microbial activity (Schlesinger, 1991). In the process of biological N fixation, atmospheric N_2 is fixed into ammonium by an enzyme called nitrogenase present in soil bacteria and blue-green algae. A few of soil-borne bacterial species form symbiotic associations with higher plants such as legumes and some woody plants. In this efficient symbiotic relationship, N-fixing bacteria provide the host plants with the fixed N and in return receive energy source (e.g. sugars) from the hosts. For this reason, legume species are often exploited in the crop rotations in conventional agricultural practices in order to increase the fixed N in the soil. However, N fixed by the natural processes is not generally sufficient for the optimal growth of crop plants.

The industrial fixation of N occurs using the Harber-Bosch process which produces ammonium from atmospheric N_2 and hydrogen, under elevated temperature and high pressure (Frink *et al.*, 1999). This reaction is of great importance to agriculture since the N fixed by this process is the starting point of all the N fertilizers, and the optimal growth of crop plants is dependent on the sufficient supply of N fertilizers. Over the past 50 years, annual worldwide consumption of N fertilizer has increased from 10.83 million metric tons (MMt) in 1960 to 90.86 MMt in 2005 (Data from International Fertilizer Industry Association, http://www.fertilizer.org/ifa/), and it is predicted to increase to 240 MMt by the year 2050 (Tilman, 1999). Currently, the amount of industrial N fixation is becoming almost equal to the total amount of biological N fixation (100–130 MMt/year), resulting in the nearly doubling of N introduced into terrestrial ecosystems.

1.1.2. Biogeochemical nitrogen cycle

Once N has been fixed into nitrate or ammonium, it enters the biogeochemical cycle (Taiz and Zeiger, 1998). The fixed N is taken up and utilized by many organisms and moves through the environment in various organic or inorganic forms, before eventually returning to atmosphere as gaseous N (Figure 1.1; Table 1.1). Biologically active forms of N, such as NO_3^-, NH_4^+ and amino acids, are released to soils as the result of natural N fixation, decomposition of organic matter and N fertilizer application. In general, nitrate is the most abundant form of N available to plants in cultivated soils. It should be noted that the other forms of N are also converted to nitrate by soil microorganisms. However, nitrate is subjected to being lost from soils before taken up by plants, through both denitrification and leaching. In the denitrification process, soil bacteria convert nitrate to nitrous oxide and molecular nitrogen; and the converted N returns to the atmosphere as gaseous forms of N. In addition, since soil particles do not adsorb negatively charged nitrate ions, nitrate is highly mobile in the soil and can leach into ground water when excess N is applied as fertilizers. Thus, nitrate and ammonium in the soils become the object of intense competition between plants and soil microorganisms, and the rate of the N acquisition by plants depends on many factors, such as soil types, species and local climate (Hodge *et al*., 2000, Kaye and Hart, 1997). In general, the proportion of the captured N by crop plants rarely exceeds 50% of the total input of N fertilizer in a typical agricultural setting (Kronzucker *et al*; 1998).

1.1.3. Impact of the excessive N fertilizer use on environment

The substantial increase in N fertilizer use over the past four decades has been one of the key factors that has resulted in the doubling of the agricultural production (Hirel *et al*., 2007). However, the environmental impact of the excessive application of agricultural N fertilizers is becoming a serious global issue (Matson *et al*., 1998). The increased nitrate leaching and the loss of N-containing gases (NO, N_2O, NH_3) to the atmosphere are resulting in the eutrophication of many terrestrial and aquatic ecosystems (London, 2005; Benman *et al.,* 2006). For example, volatilization of NH_3 from the agricultural soils facilitates the movement of the applied N into surrounding environment, influencing the population density and diversity of microorganisms, plants and animals in the affected ecosystems (Mosier, 2001). One of the effects of the increased leaching of nitrates into freshwater and marine ecosystems has been the enhanced growth of algal species in the N-enriched environment. This can create an oxygen deficient zone in the water and cause the death of fish inhabiting the ecosystem (Smith 2003).

Due to the high cost associated with N fertilizer use and its detrimental impacts on the environment, the need to reduce agricultural N inputs and, yet maintaining the high yield at the same time, is now well recognized (Lea and Azevedo, 2007). This goal may be achieved through the more efficient management of fertilizer application by matching the timing of the fertilizer input to the crop growth cycle, and through the development of nitrogen efficient cultivars by conventional breeding program and/or transgenic approaches (Matson *et al*., 1998; Good *et al*., 2004; Hirel *et al*., 2007).

1.2. Plant Nitrogen Metabolism

As N nutrition is the principal limiting factor for plant growth and development in most natural and agricultural settings, N uptake and its subsequent assimilation in plants has been the subject of intensive research for many decades. To date, the molecular mechanism of plant N metabolism has been studied using a number of physiological, biochemical and genetic studies. Recent advances in the functional genomics in model plant species (i.e. rice and *Arabidopsis*) allowed us to identify and dissect the individual component of this complex metabolic process. However, despite the many significant research advances, the basics of how plants monitor their internal N status and how they take up and transport N still remain to be elucidated. In this section, N uptake, ammonium assimilation and the subsequent amino acid metabolism are discussed along with the recent progresses made in these research areas.
(See Appendix 1 for specific reactions of the related enzymes).

1.2.1. Nitrate uptake

Nitrate is the major source of N available to crop plants in cultivated soils. Therefore, the uptake of nitrate is usually the first step of N utilization in plants. This process requires the transport of nitrate across the plasma membrane at the root epidermal and cortical cells. The nitrate influx is mediated by proton co-transporters and is energized by the proton-motive force across the membrane (Miller *et al*., 2007a). Physiological investigations on nitrate uptake have led to the conclusion that plants employ three types of transport systems in order to effectively take up nitrate at a wide range of the external concentrations (Crawford and Glass, 1998; Forde, 2000). A constitutive high-affinity transport system (cHATS) and an inducible high-affinity transport system (iHATS) typically operate at low nitrate concentration (< 1 mM). On the other hand, a low-affinity transport system (LATS) largely contributes to the nitrate uptake at the concentrations above 1 mM. However, recent studies have suggested that the classification into the two systems, high- and low- affinity transport systems, is rather vague. For instance, both cHATS and iHATS can contribute to the nitrate acquisition at the concentrations above 1 mM (Miller *et al*., 2007a).

A number of the components involved in the nitrate transport systems have now been identified, including genes encoding nitrate transporters which have been isolated from the fungus, *Aspergillus nidulans* (Unkles *et al.*, 1991), and *Arabidopsis* (Tsay *et al.*, 1993), both of which were discovered by the screen for the resistance to herbicide, chlorate. Currently, the identified nitrate transporters (NRTs) were categorized into two groups, the NRT1 and NRT2 families. In *Arabidopsis*, 53 members were found for NRT1 family; however, this group includes amino acid and peptide transporters along with nitrate transporters (Miller *et al.*, 2007a). By contrast, 7 members were found for NRT2 family (Miller *et al.*, 2007a).

In terms of the molecular identity of LATS, AtNRT1.1, originally identified as CHL1, was thought to be the candidate transport protein for LATS since a reduction in low affinity nitrate uptake was observed in the *Arabidopsis chl1* mutant (Tsay *et al.*, 1993). However, the deficiency in low affinity uptake was only evident when grown on mixed nitrate and ammonium supplies, indicating the presence of another LATS operating when plants were grown on nitrate as the sole N source (Touraine and Glass, 1997). Although the involvement of AtNRT1.1 in LATS has been repeatedly confirmed in subsequent studies, the nitrate-inducible feature of AtNRT1.1 is in contrast to the characteristic of LATS as a constitutive uptake system (Huang *et al.*, 1999; Okamoto *et al.*, 2003). In addition, AtNRT1.1 has also been suggested to be a dual-affinity transporter depending on the phosphorylation state of the protein (Liu and Tsay, 2003). By contrast, *AtNRT1.2* is constitutively expressed in the root epidermis, and the heterologous expression of AtNRT1.2 in *Xenopus* oocytes has been demonstrated to display a low affinity transport activity (Huang *et al.*, 1999). However, the reduction in LATS activity in the antisense lines of *AtNRT1.2* did not correlate with the decrease in the expression of *AtNRT1.2* (Huang *et al.*, 1999). Taken together, both AtNRT1.1 and AtNRT1.2 are likely involved in LATS, but their roles seem more complicated than once envisioned.

The elucidation of the molecular basis for HATS has resulted from the identification and characterization of *NRT2* homologs in several plant species. These homologs were found based on the sequence homology to the nitrate transporter gene, *CRNA*, identified in *Aspergillus nidulans* (Unkles *et al.*, 1991). The *Arabidopsis* counterparts of CRNA, AtNRT2.1 and AtNRT2.2, have been demonstrated to be the major components of HATS since HATS activity was impaired when *AtNRT2.1* and *AtNRT2.2* were mutated (Filleur *et al.*, 2001; Orsel *et al.*, 2004). Interestingly, the heterologous expression of NRT2 homologs in *Xenopus* oocyte required the co-expression of another protein, NAR2, for the nitrate uptake activity, indicating the involvement of the two proteins for HATS activity (Zhou *et al.*, 2000; Tong *et al.*, 2005; Orsel *et al.*, 2006). The two-component high affinity uptake system has been recently demonstrated through the analyses of *AtNAR2.1* mutants, which showed reduced HATS activity similar to the mutants of *AtNRT2.1* (Okamoto *et al.*, 2006; Orsel *et al.*, 2006). Furthermore, direct protein-protein interaction of AtNRT2.1 and AtNAR2.1 has been confirmed, and the role of AtNAR2.1 in targeting NRT2 proteins to the plasma membrane been proposed (Okamoto *et al.*, 2006; Orsel *et al.*, 2006; Miller *et al.*, 2007a).

The regulation of nitrate uptake activity is directly induced by nitrate. Also, nitrate uptake is repressed by the external supply of amino acids, most strongly by glutamine (Miller *et al.*, 2008). This regulation in accordance to the N supply indicates the presence of a mechanism that coordinates the nitrogen uptake process with cellular N-status. For instance, expression of *AtNRT2.1* is induced by nitrate, but it is also regulated through the feedback repression exerted by cellular N metabolites, such as ammonium and amino acids (Zhuo *et al.*, 1999; Nazoa *et al.*, 2003; Miller *et al.*, 2007a). Moreover, the activity of these transport systems is likely controlled by post-translational events, including phosphorylation and the interaction with 14-3-3 proteins (Miller *et al.*, 2008). In terms of sensing the available nitrate levels at the root surface, it has been suggested that AtNRT2.1 is directly involved in nitrate sensing and functions as a negative regulator for the initiation and elongation of lateral roots (Little *et al.*, 2005). The role of AtNRT1.1 in regulating the expression of *AtNRT2.1* and in the induction of lateral root formation in the nitrate-rich areas has also been reported (Munos *et al.*, 2004; Krouk *et al.*, 2006; Remans *et al.*, 2006). Nevertheless, a more detailed picture of the regulation of nitrate uptake and the mechanism of nitrate sensing has yet to be uncovered. Clearly, understanding this area of research will be important in improving the N acquisition of crop plants and possibly in reducing the input of agricultural N fertilizers.

1.2.2. The reduction of nitrate and ammonium formation

Nitrate taken up by plant roots is reduced to nitrite (NO_2^-) by nitrate reductase (NR) in the cytosol. The NR in most plants uses NADH as a reductant to carry out the reduction of nitrate, however a bispecific-NR that uses NADPH as a reductant has been also reported (Sueyoshi *et al.*, 1995). Campbell (1996) has described the structure of NR; in summary, active NR comprises two identical subunits and contains a molybdenum cofactor, Fe, heme and FAD. Nitrite, the product of the reaction catalyzed by NR, is potentially toxic to plant cells. Therefore, the nitrite formed is rapidly converted to ammonium by nitrite reductase (NiR) in root plastids or in leaf chloroplasts. Active NiR functions as a monomer and requires reduced ferredoxin (Fd), a 4Fe-4S cluster and siroheme for its activity (Dose *et al.*, 1997).

After the genes and proteins corresponding to NR and NiR activity were identified in plants, the transcriptional and post-translational regulation of NR and NiR activity became the subject of the intensive research. In higher plants, NR and NiR activities are tightly controlled by various environmental factors and endogenous signals (Lilo *et al.*, 2004). Among the key positive regulators for both enzymes are light and nitrate. In fact, the steady-state mRNA levels of both NR (*Nia*) and NiR (*Nii*) genes increases in response to light and nitrate, and the levels of the enzyme activity often show a diurnal oscillation according to the light cycle (Crawford, 1995; Vincentz *et al.*, 1993). The post-translational inactivation of NR by phosphorylation and the subsequent binding of 14-3-3 proteins play an important role in preventing the accumulation of toxic nitrite in the conditions where NiR activity is restricted (Lilo *et al.*, 2004). Indeed, the post-translational regulation of NR has been recently demonstrated to be more important than the transcriptional regulation of *Nia* expression (Lea *et al.*, 2006). In their study, NR activity was deregulated at the transcriptional level by the over-expression of a *Nia* gene under the control of the CaMV 35S promoter and at the posttranslational level by the mutation of the regulatory phosphorylation site, serine 521 (line S521D). The loss of the phosphorylation site transformed the mutated NR into the constitutively active NR (Lea *et al.*, 2006). As a result, the reduction in NR activity that generally occurs during the dark period was abolished in the S521D line, and this lead to a large increase in the rate of nitrate assimilation in dark conditions (Lea *et al.*, 2006). Regarding the regulation of

NiR, the post-transcriptional regulation exerts a stronger effect on NiR activity than the transcriptional regulation. When the *Nii* gene was constitutively expressed using the CaMV 35S promoter, increased NiR activity was observed only on nitrate-containing media but not on ammonium-containing media (Crété *et al.*, 1997). Since NiR proteins appear to be stable even in the dark, the requirement of nitrate for the increase in the NiR activity suggests the involvement of nitrate, nitrite or the nitrate-mediated signal for the translation of *NiR* transcripts (Crété *et al.*, 1997). Taken together, the process of reduction of nitrate to form ammonium appears strongly regulated at the post-transcriptional and post-translational levels, and hence, the improvement of the nitrate assimilation process has proved to be difficult simply through the modulation of NR and NiR gene expression. It would require the controlled regulation of NR and NiR at post-transcriptional and post-translational levels in order to improve the nitrate and nitrite reduction in crop plants.

1.2.3. Ammonium uptake

In addition to nitrate (and nitrite for a lesser extent), plant roots take up ammonium as an N source. The reduced state of ammonium N makes it attractive as a nitrogenous nutrient for plants (Loqué and Wirén, 2004). However, controlling the fluxes of ammonium across the membrane is difficult since ammonium exists in equilibrium between NH_4^+ and NH_3, and the latter uncharged form is highly permeable to membranes. On the other hand, the transport of NH_4^+ is mediated by several membrane proteins (Loqué and Wirén, 2004). Evidence from electrophysiological studies has indicated that the mechanism of ammonium uptake is due to a uniport for NH_4^+, depending on the membrane potential. (Ludewig *et al.*, 2002; Loqué and Wirén, 2004). Moreover, similar to the systems for nitrate uptake, low-affinity transport and high-affinity transport systems for ammonium are operative in plants (Forde and Clarkson, 1999).

The first identified ammonium transporter in plants, *AtAMT1;1* was isolated by a functional complementation of yeast mutant defective in ammonium uptake (Ninnemann *et al.*, 1994). Subsequent to the completion of *Arabidopsis* genome sequencing, another five genes were identified based on the sequence homology to *AtAMT1;1* (Loqué and Wirén, 2004). The transcriptional regulation of these *Arabidopsis AtAMT* genes has been recently studied (Engineer and Kranz, 2007). Their results demonstrated that the members of *AtAMT1* family of genes are expressed in roots, conversely *AtAMT2;1* is more strongly expressed in leaves. Among the five transcripts of the *AtAMT* family that were detected, the *AtAMT1;1* expression pattern was unique in that induction was reciprocal between roots and leaves depending on N availability. The strong up-regulation of *AtAMT1;1* in roots under N starvation suggests that its role is in scavenging available ammonium in the rhizosphere, consistent with its function in high-affinity transport system, while the induction in leaves under sufficient N supply indicates its involvement in the import of ammonium into xylem parenchyma cells or leaf mesophyll cells (Engineer and Kranz, 2007). The role of AtAMT1;1 in leaves was supported by the phenotype observed in *atamt1;1* mutants where the mutant line showed the thickening of mesophyll tissues and a decrease in the intercellular airspace between mesophyll cells (Kaiser *et al.*, 2002). Moreover, constitutive expression of *AtAMT1;1* by the CaMV 35S promoter in tobacco and *Arabidopsis* suggested that mRNA turnover is important for regulating the levels of ammonium transporters in response to the N-status of plants (Yuan *et al.*, 2007). In addition to N availability, light and sugar levels increase the steady-state levels of mRNA for *AMT* genes, such as *AtAMT1;1*, 1;2 and *1;3* (Lejay *et al*, 2003; Loqué and Wirén, 2004; Engineer and Kranz, 2007). Elucidating the molecular basis of ammonium fluxes in plants is important not only for the improvement of nitrogen acquisition in crop plants but also for understanding the ammonium toxicity and conferring tolerance to high ammonium levels.

1.2.4. Ammonium assimilation: glutamine synthetase/ glutamate synthase cycle

The incorporation of ammonium into amino acids is facilitated by the concerted action of glutamine synthetase (GS) and glutamate synthase (GOGAT, also known as glutamine 2-oxoglutarate amidotransferase). This reaction, the so-called GS/GOGAT cycle, constitutes the sole entry port of inorganic N into the organic form, and thus, this reaction plays the central role in plant N metabolism (Lam *et al.*, 1996; Lea and Ireland, 1999; Miflin and Habash, 2002; Suzuki and Knaff, 2005). GS catalyzes the addition of ammonium to glutamate to form glutamine at the expense of ATP, while GOGAT produces two molecules of glutamate from glutamine and 2-oxoglutarate provided there is a source of reductant.

Ammonium assimilation through GS/GOGAT cycle was first demonstrated by Lea and Miflin in 1974. Before then, glutamate dehydrogenase (GDH) had been thought to be responsible for the incorporation of ammonium into glutamate in higher plants. For this reason, the discovery of the GS/GOGAT cycle has created a long debate over the role played by GDH. It has been suggested that GDH represents an alternative route for the ammonium assimilation. In fact, this possibility has been repeatedly investigated, and the relative contribution of the GS/GOGAT cycle and GDH in ammonium assimilation has been compared in many studies in the past (reviewed by Lam *et al.*, 1996; Lea and Ireland, 1999). For instance, in labeled N tracer studies, the pattern of $^{15}NH_4^+$ incorporation into the amide and amino groups of glutamine as well as the incorporation into the amino group of glutamate was carefully monitored, and the rate of ammonium assimilation by GDH was concluded to be minuscule compared to the total rate of N assimilation (Rhodes *et al.*, 1980, 1989). When the incorporation of $^{15}NH_4^+$ was followed in an NMR study, the labeled N was observed in the amide position of glutamine during the first 30 min and in the amino position in glutamate during the subsequent hours, demonstrating the ammonium assimilatory function of the GS/GOGAT cycle (Robinson *et al.*, 1991). In the studies using inhibitors, two GS inhibitors, methionine sulfoximine (MSX) and phosphinothricin (PPT) have been commonly used to investigate the role of GS in ammonium assimilation. In these experiments, treatment with either MSX or PPT generally blocked ammonium assimilation and caused the accumulation of free ammonium ions (reviewed by Lea and Ireland, 1999), emphasizing the importance of GS as the sole path of ammonium incorporation into glutamine/glutamate. Azaserine, an inhibitor for GOGAT, has been also used in some experiments. For example, the application of azaserine resulted in the accumulation of ^{15}N-labeled glutamine and prevented glutamate formation (Monselise and Kost, 1993; Aarnes *et al.*, 1995), demonstrating the chief role of GOGAT in glutamate synthesis. Furthermore, both mutants for chloroplastic GS (Blackwell *et al.*, 1987; Wallsgrove *et al.*, 1987) and Fd-dependent GOGAT (Somerville and Ogren, 1980; Kendall *et al.*, 1986, Blackwell *et al.*, 1988) exhibited impaired growth under normal conditions but recovered normal growth under non-photorespiratory high CO_2 concentrations (reviewed by Leegood *et al.*, 1995; Lea and Ireland, 1999). These results suggest that the concerted operation of chloroplastic

GS and Fd-dependent GOGAT assimilates ammonium generated by the decarboxylation of glycine in the mitochondria during photorespiration (Leegood *et al.*, 1995). Moreover, the phenotype of the mutants deficient in chloroplastic GS or Fd-GOGAT rejects the potential role of GDH, localized in mitochondria, in the assimilation of photorespiratory ammonium. As many studies have indicated, it is now generally accepted that GS/GOGAT cycle is the major, if not only, reaction responsible for ammonium assimilation in plants, while GDH plays a negligible role in this process (Miflin and Habash, 2002; Dubois *et al.*, 2003).

In terms of the isoenzymes for GS, cytosolic and chloroplastic forms of GS have been identified and are currently known as GS1 and GS2, respectively. In plants, chloroplastic GS2 is encoded by a single gene, while cytosolic GS1 is encoded by a small gene family containing up to 5 members (*Arabidopsis* and maize; Ishiyama *et al.*, 2004; Martin *et al.*, 2006). The major function of GS2 is the assimilation of photorespiratory ammonium, as suggested by the photorespiration-deficient phenotype of *GS2* mutants, whereas cytosolic GS1 appears to be important in the primary N assimilation, supported by the normal growth of *GS2* mutants under non-photorespiratory conditions (Tabuchi *et al.*, 2007). In recent studies, the specific functions of each member of the GS1 family have been characterized by biochemical, transcriptional and mutational analyses. In *Arabidopsis*, the members of the GS1 family, *GLN1;1, GLN1;2, GLN1;3, GLN1;4* was investigated (Ishiyama *et al.*, 2004) (*GLN1;5* was not studied as its expression was not detected). The kinetic properties of the GS1 isoenzymes revealed by analysing the recombinant GS1 proteins expressed in *E. coli* demonstrated the presence of distinct GS1 isoenzymes with high- (GLN1;1 and GLN1;4) and low-affinity for ammonium (GLN1;2 and GLN1;3) (Ishiyama *et al.*, 2004). The different expression patterns of the *GLN1* genes also indicated the specific function of each GS1 isoenzyme; for instance, the expression of *GLN1;1* in the surface layer of roots under N starvation suggested its involvement in glutamine formation in the root surface cells and the promotion of N uptake, while the up-regulation of *GLN1;2* in the vasculature by exogenous ammonium supply demonstrated the participation of GLN1;2 in glutamine synthesis in those tissues, under excess N conditions (Ishiyama *et al.*, 2004). These distinct characteristics of the GS1 isoenzymes may contribute to the homeostatic control of glutamine synthesis in

Arabidopsis (Ishiyama *et al*., 2004). In rice, 3 GS1 homologs, *OsGS1;1*, *OsGS1;2* and *OsGS1;3* have been identified. The expression of *OsGS1;1* was observed in roots, leaf blades, leaf sheaths and spikelets with higher expression in leaf blades, and that of *OsGS1;2* was mainly found in roots and spikelets. *OsGS1;3* expression was only detected in spikelets (Tabuchi *et al*., 2005). In addition, mutants defective in *OsGS1;1* were identified and these showed severe retardation in growth rate and grain filling under the normal N regime, demonstrating the non-redundant role of *OsGS1;1* in primary N assimilation (Tabuchi *et al*., 2005). Furthermore, the specific roles of two GS1 isoenzymes in maize were also characterized recently by analyzing *gln1-3* and *gln1-4* mutants and the *gln1-3/gln1-4* double mutant (Martin *et al*., 2006). In these mutants, the vegetative growth was not affected, but the kernel yield was significantly reduced (Martin *et al*., 2006). This phenotype suggested the specific role of GS1-3 and GS1-4 in controlling kernel production in maize; in fact, this conclusion was further supported by the over-expression of *GLN1-3*, which resulted in the increase in the kernel yield (Martin *et al*., 2006). The expression of GS1-3 in leaf mesophyll cells and that of GS1-4 in leaf bundle sheath cells indicated the importance of the translocation of N metabolites (mainly glutamine and asparagines) from senescing leaves to the developing kernels (Martin *et al*., 2006). Interestingly, in genetic mapping studies, *GS1* loci (both *GSN1-3* and *GSN1-4*) have been shown to be coincident with a QTL for kernel production (Hirel *et al*., 2001), further substantiating the proposed role of leaf GS1 in kernel productivity. With respect to the specific roles of other GS1 isoenzymes, proposed roles for maize GS1 isoenzymes were suggested based on the tissue-specific expression patterns; for example, the role of root-specific GS1-1 (also called GS1r) was suggested to be in the synthesis of glutamine for vegetative growth (Sakakibara *et al*., 1992; Martin *et al*., 2006).

Regarding GOGAT isoenzymes, the two different forms that are present in plastids vary in their reductant specificity; Fd-dependent GOGAT (Fd-GOGAT) and NADH-dependent GOGAT (NADH-GOGAT). Typically, the activity of Fd-GOGAT accounts for the majority of GOGAT activity in leaves, and its involvement in the assimilation of photorespiratory ammonium has been demonstrated in Fd-GOGAT-deficient mutants of *Arabidopsis* (Somerville and Ogren; 1980; Ireland and Lea; 1999). In *Arabidopsis*, two Fd-GOGAT genes (*GLU1* and *GLU2*) and one NADH-GOGAT gene

(*GLT1*) have been identified (Coschigano *et al*., 1988; Lancien *et al*., 2002). The expression of *GLU1* was more restricted in shoots and is subject to regulation by light and sucrose, while that of *GLU2* and *GLT1* was higher in roots than in leaves (Coschigano *et al*., 1988; Lancien *et al*., 2002). This difference in expression pattern indicates the specific role of each GOGAT isoenzyme in plants. In fact, characterization of a mutant deficient in NADH-GOGAT, *glt1-T*, demonstrated a non-redundant role of GLT1 in ammonium assimilation as suggested by the defects in growth and glutamate biosynthesis under non-photorespiratory conditions (Lancien *et al*., 2002). Moreover, the overlap in the expression of NADH-GOGAT with that of cytosolic GS1 supports the model of an effective operation of the GS/GOGAT cycle (Tobin and Yamaya, 2001; Lancien *et al*, 2002). In respect to the translocation of N metabolites, it has been suggested that in rice, NADH-GOGAT is expressed in sink organs, such as young leaves and developing grains, and is important in the reutilization of transported glutamine synthesized by GS1 in senescing leaves (Hayakawa *et al*., 1994; Tabuchi *et al*., 2007).

Because of the central role played by the GS/GOGAT cycle in plant N metabolism, several attempts have been made to improve the nitrogen economy of plants through the augmentation in GS1 expression (reviewed by Good *et al*., 2004; Tabuchi *et al*., 2007). These include studies in *Lotus corniculatus* (Vincent *et al*., 1997), poplar (Gallardo *et al*., 1999), tobacco (Feuntes *et al*., 2001; Oliveira *et al*., 2002), alfalfa (Ortega *et al*., 2001) and maize (Martin *et al*., 2006). However, the effect of GS1 over-expression varied among these experiments and has not necessarily linked to improved plant growth. As demonstrated by the studies discussed above, each GS1 isoenzyme differs in expression pattern and enzyme kinetics and thus plays a distinct role in N metabolism in plants. The transcriptional modulations of specific GS1 isoenzyme may alter a specific aspect of plant growth and development as in the case of the over-expression of *GLN1-3* in maize, where grain yield increased without any increase in shoot dry matters (Martin *et al*., 2006). In addition, the tissue-specificity and timing of GS1 expression would be important to maximize the effect of GS1 over-expression on the growth and yield of crop plants. The importance of the coordination of the GS/GOGAT step with the carbon (C) metabolism should not be overlooked in order to achieve improved plant growth since ammonium assimilation requires C skeletons as N acceptors (Hirel *et al.*, 2007).

1.2.4. Metabolism of glutamate: The synthesis of other amino acids

Glutamate is the precursor for all other amino acids and thus occupies the central position in amino acid metabolism in plants (Forde and Lea, 2007). Once ammonium is assimilated into glutamate via GS/GOGAT cycle, the α-amino group of glutamate can be transferred to other amino acids by transamination (Figure 1.2; Lea and Ireland, 1999; Forde and Lea, 2007). Figure 1.3 depicts the C flow in the amino acid biosynthesis. Although transamination reactions were omitted in Figure 1.3, the amino group of glutamate is used for the biosynthesis of other amino acids. In addition, it should be noted that both the C skeletons and the α-amino group of glutamate are used for the synthesis of γ-aminobutyric acid (GABA), arginine and proline (Forde and Lea, 2007).

The transfer of the α-amino group from glutamate to other amino acids is catalyzed by aminotransferases (also referred to as transaminases). Aminotransferases catalyze the transfer of the α-amino group from a donor amino acid to the recipient 2-oxo acid, producing a new amino acid and concomitantly degrading the amino donor to a 2-oxo acid (Figure 1.2; Liepman and Olsen, 2004). This reaction is generally reversible and requires pyrydoxal-5'-phosphate as an essential cofactor (Liepman and Olsen, 2004). Aminotransferase are also known to show a wide range of substrate specificity, so it has been frequently observed that one aminotransferase catalyzes several transaminations with various substrate pairs. However, these observations are often based on older studies where it has been difficult to purify the specific aminotransferases. Aminotransferases are found in various subcellular compartments and participate in the diverse metabolic processes such as amino acid synthesis, nitrogen redistribution, synthesis of secondary metabolites and photorespiration (Lea and Ireland, 1999; Liepman and Olsen, 2004).

Although glutamate is used for the synthesis of virtually all amino acids, the synthesis of asparagine is particularly important with regard to N transport and storage in plants. In general, asparagine and glutamine constitute the major components of organic N in both xylem and phloem sap, although there is a wide range of variations in the form of N transported in xylem dependent on species (Lea and Ireland, 1999). These amides play important roles in the distribution of N assimilated by the GS/GOGAT cycle in various parts of a plant. In addition, asparagine is used as an N storage compound, because of its stability and the high N/C ratio (2N to 4C for asparagine versus 2N to 5C for glutamine). For example, conditions leading to high carbohydrate levels (i.e. high light) stimulate nitrate uptake, nitrate reduction and glutamine/glutamate formation by GS/GOGAT cycle, but inhibit asparagine synthesis, favoring the production of compounds that are rich in carbon. In contrast, conditions leading to low carbohydrate levels repress the processes of glutamine/glutamate formation but stimulate the production of asparagine, a sufficiently stable compound that is rich in N (Lea *et al.*, 2007).

In the synthesis of asparagine, nitrogen assimilated into glutamate is first transferred to oxaloacetate to form aspartate by the action of aspartate aminotransferases (AAT). Aspartate is the precursor for other amino acids, lysine, threonine, methionine and isoleucine, and hence, aspartate synthesis is the key step in the metabolism of the aspartate family of amino acids (Figure 1.3; Azevedo *et al.*, 2006). In plants, several AAT isoenzymes are present in cytosol, mitochondria, plastids and peroxisomes (Liepman and Olsen, 2004). For instance, five genes encoding AAT polypeptides have been identified in *Arabidopsis; ASP1* (mitochondrial), *ASP2* and *ASP4* (cytosolic), *ASP3* (peroxisomal) and *ASP5* (plastidic) (Schultz and Coruzzi, 1995; Liepman and Olsen, 2004). Furthermore, genes encoding prokaryotic-type AAT have been recently identified and their potential roles in amino acid metabolism in the chloroplasts of photosynthetic tissues have been suggested (Torre *et al.*, 2006). Subsequent to the aspartate formation, asparagine is synthesized through the glutamine-dependent amidation of aspartate, which is catalyzed by asparagine synthetase (AS). In *Arabidopsis*, a small gene family (*ASN1*, *ASN2* and *ASN3*) encodes AS, and the reciprocal expression pattern of *ASN1* and *ASN2* is regulated by light and metabolites (Wong *et al.*, 2004). In short, *ASN1* expression is up-regulated by dark but repressed by light, while *ASN2* expression is up-regulated by light in an ammonium-dependent manner (Wong *et al.*, 2004). This reciprocal regulation of *ASN1* and *ASN2* suggests that the accumulation of asparagine in the dark is mediated by ASN1 in *Arabidopsis* (Wong *et al.*, 2004). In addition, the over-expression of *ASN1* resulted in the increased deposit of asparagine into reproductive organs, as indicated by the increase in asparagine content in phloem exudates, increase in total seed proteins and the enhanced tolerance of young seedlings to nitrogen limitation (Lam *et al*., 2003). These results clearly demonstrate the important role of asparagine in the N transport and storage.

1.2.5. Alanine in plants

Alanine is generally not considered to be one of the major products of primary N assimilation in plants. However, many classical labeling studies have shown that significant amounts of labeled N are incorporated into alanine (Thorpe *et al*, 1989; Magalhães *et al*., 1990; Robinson *et al*., 1991; Amancio and Santos 1992). In addition, this incorporation into alanine becomes the major route of N assimilation under low-oxygen stress conditions (Vanlerberghe *et al*., 1991; Good and Muench, 1993; Reggiani and Bertani, 2003). Nonetheless, the role of alanine synthesis in plant N metabolism is unclear. The metabolism of alanine is further complicated by the involvement of several aminotransferases that catalyze the synthesis and/or breakdown of alanine.

Alanine aminotransferase (AlaAT) is an important enzyme involved in the metabolism of alanine. The enzyme catalyzes the reversible transfer of amino group from glutamate to pyruvate, thereby producing 2-oxoglutarate and alanine. AlaAT enzyme activities have been found in the cytosol, mitochondria and peroxisomes, and several isoenzymes of AlaAT have been reported (Liepman and Olsen, 2004). One of the better-defined functions of AlaAT is its involvement in C_4 photosynthesis where AlaAT mediates the aspartate/alanine shuttle between mesophyll and bundle-sheath cells (Figure 1.4; Hatch, 1992; Leegood, 1997). In support for this function, AlaAT has been studied in a C_4 plant, broomcorn millet (*Panicum miliaceum*), and the light- and nitrogen-dependent regulation of the *AlaAT* as well as its expression in both mesophyll and bundle-sheath cells have been demonstrated (Son *et al*.,1991; 1992; Son and Sugiyama, 1992). Moreover, the function of AlaAT appears to be critical in the metabolism of alanine under low-oxygen stress conditions (covered in more detail in Chapter 1.4). This was evident by the induction of *AlaAT* expression under low-oxygen conditions that has been reported in several species (Good and Muench, 1993; Muench *et al*., 1998; Ricoult *et al*., 2005). In respect to the function of AlaAT in the primary N assimilation, its important role has been suggested by the growth improvement of transgenic canola (*Brassica napus*) expressing barley *AlaAT* under the control of a stress inducible promoter, *btg26* (Good *et al*., 2007). Although the molecular basis of this improved growth of the transgenic canola is uncertain, the results clearly demonstrate that alanine is a key amino acid in the primary N assimilation process.

Besides the AlaAT reaction described above, the isoenzymes of AlaAT localized in peroxisomes are also capable of catalyzing the amino group transfer from glutamate to glyoxylate, forming 2-oxoglutarate and glycine (Liepman and Olsen, 2003; Igarashi *et al*., 2003). These isoenzymes are alternatively called glutamate:glyoxylate aminotransferase (GGAT). GGAT normally facilitates glycine synthesis *in vivo* and plays a key role in the photorespiratory pathway (Figure 1.5; Liepman and Olsen, 2003; Igarashi *et al*., 2003). Interestingly, another photorespiratory aminotransferase (AGT1) that catalyzes the serine:glyoxylate transamination may be involved in the metabolism of alanine through the serine:pyruvate transamination (Figure 1.5; Liepman and Olsen, 2001). In the photorespiratory pathway, AGT1 is responsible for the conversion of serine into hydroxy-pyruvate *in vivo* (Liepman and Olsen, 2001). *Arabiodpsis* mutants defective in *GGAT1* and *AGT1* both exhibited a typical photorespiration-deficient phonotype, which is the severe growth retardation in air but the phenotypic recovery under non-photorespiratory conditions (Liepman and Olsen, 2001; Igarashi *et al*., 2003). Although alanine does not seem to be involved in the photorespiration at first glance, many studies have shown that photorespiration affects the metabolism of alanine. In general, glycine and serine are positively correlated with the rate of photorespiration, while alanine and aspartate are negatively correlated (Novitskaya *et al*., 2002; Foyer *et al*., 2003). It is likely that the amino groups of alanine and aspartate are used to produce glycine and serine during photorespiration (Novitskaya *et al*., 2002; Igarashi *et al*., 2006). Supporting this, it has been demonstrated by labeling studies that the amino group of alanine was used in the synthesis of glycine and serine during photorespiration (Betsche, 1983; Betsche and Eising, 1986). In order to transfer the amino group from alanine to glycine, the amino group of alanine may be first transferred to glutamate by GGAT and/or AlaAT then subsequently transferred to glycine by GGAT (Novitskaya *et al*., 2002; Igarashi *et al*., 2006). Although the precise role of alanine in photorespiration is still ambiguous, these aminotransferases play a key role in the amino acid metabolism in the photorespiration-active tissues.

1.2.6. GABA in plants

GABA is a ubiquitous four-carbon, non-protein amino acid. The role of GABA in plants is rather vague, in contrast to the well-defined role of GABA in neurotransmission in animals (Shelp *et al.*, 1999; Bouché and Fromm, 2004). Although this amino acid has been considered as a metabolite which is accumulated with the onset of biotic and abiotic stresses, the role of GABA as a signaling molecule has been shown in recent studies (reviewed by Bouché and Fromm, 2004).

The metabolism of GABA is mediated by three enzymes; glutamate decarboxylase (GAD), GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH). The GABA metabolism pathway facilitating the conversion of glutamate to the succinate is called the GABA shunt since this short pathway bypasses the two steps of the TCA cycle (Figure 1.6; Shelp et al. 1999; Bouché and Fromm 2004). Generally, GABA is synthesized from glutamate in a reaction catalyzed by GAD. After GABA is transported into the mitochondria by as yet unidentified transporter(s), GABA is converted to succinic semialdehyde (SSA) by GABA-T. This transamination transfers the amino group from GABA to either pyruvate or 2-oxoglutarate, producing alanine or glutamate, respectively. The enzyme catalyzing the former reaction is specifically called GABA-TP (or simply GABA-T in the remainder of this thesis), while the latter is called GABA-TK (K for α-ketoglutarate). Only GABA-TP has been identified to date in plants (Van Cauwenberghe *et al.*, 2002). Subsequent to the GABA-T reaction, SSA is oxidized to succinate by SSADH, thereby completing the GABA shunt pathway.

As the GABA shunt mediates the connection between C and N metabolism, this pathway can contribute to the adjustment of the C/N balance in plants. The evidence of GABA metabolism controlling glutamate levels was provided by the over-expression of a constitutively active GAD that resulted in the decrease in glutamate levels (Baum *et al.*, 1996). In addition, GABA synthesis is increased under the conditions that inhibit glutamine synthesis, reduce protein synthesis or enhance protein degradation (reviewed by Shelp *et al.*, 1999). GABA synthesized under such conditions may have a function as a temporal N storage (Shelp *et al.*, 1999).

The accumulation of GABA has been observed in plants in response to various stresses, including heat shock, cold, hypoxia, and mechanical wounding (Shelp *et al.*, 1999; Bouché and Fromm, 2004). The increase in GABA synthesis upon exposure to these stimuli appears to be mediated by the activation of GAD (Shelp *et al.*, 1999; Bouché and Fromm, 2004). Since acidic pH and Ca^{2+}/calmodulin binding to the enzyme significantly increases the activity of GAD, cytosolic acidification as well as the rise in cellular Ca^{2+} concentration induced by these stimuli triggers the rapid activation of GAD and the GABA accumulation in plant cells (Aurisano *et al.*, 1995b; Cholewa *et al.*, 1997; Shlep *et al.*, 1999; Bouché and Fromm, 2004; Bown *et al.*, 2006). Although the advantage of GABA synthesis and accumulation under these stress conditions is unclear, possible roles of GABA and the GABA shunt have been speculated (reviewed by Bouché and Fromm, 2004). For example, as the GAD reaction consumes a proton, GABA synthesis may ameliorate the effect of cytosolic acidification (discussed in Chapter 1.4). Under salt and dehydration conditions, GABA may act as an osmoprotectant in a similar manner to proline (Shlep *et al.*, 1999). Furthermore, the role of GABA as an invertebrate pest deterrent has been demonstrated by the increased resistance to the root-knot nematode and tobacco budworm larvae of transgenic tobacco containing elevated GABA levels (Maclean *et al.*, 2003; MacGregor *et al.*, 2003). It has been suggested that the wounding due to herbivory and crawling by insect larvae stimulates GABA synthesis through the activation of GAD, by the disruption of cellular compartmentation (i.e. vacuole) and the consequent decrease in cytosolic pH (Bown *et al.*, 2006). GABA may interfere with the GABA-regulated invertebrate neuromuscular junctions when ingested by insects, thus playing a role in the defense against invertebrate pests (Bown *et al.*, 2006). On the other hand, *Arabidopsis ssadh* mutant showed increased susceptibility to UV light (Bouché *et al.*, 2003). This demonstrates the role of GABA shunt in preventing the accumulation of reactive oxygen intermediates and in bypassing the steps of the TCA cycle (reaction catalyzed by α-ketoglutarate dehydrogenase) that are more sensitive to oxidative stress conditions (Bouché *et al.*, 2003). Another characterized mutant defective in the GABA shunt, suggests the possible role of GABA as a signaling molecule.

Arabidopsis pollen-pistil-interaction2 (*pop2*) mutants deficient in GABA-T activity exhibited reduced seed production (Palanivelu *et al.*, 2003). This study suggested that the GABA gradient in the pistil, which was disrupted in *pop2* mutant, is essential for the successful guidance of pollen to egg (Palanivelu *et al.*, 2003).

1.3. The transport of amino acids in plants

The distribution of N metabolites throughout the plant is another important aspect of plant N metabolism. Although the identities of the components that carry out this function had not been known in plants for many years, the use of genomic tools in model species (mostly *Arabidopsis*) led to the identification of many transporters that facilitate the movement of amino acids across membranes. In this part, the transport of amino acids in plants is discussed.

1.3.1. Plant amino acid transporters.

Once inorganic N is assimilated in either in roots or leaves, N is distributed throughout the plant via the xylem and phloem in the form of amino acids. The distribution of amino acids within the plant requires several transport steps across the membrane. For example, for the amino acids synthesized in roots to be translocated to shoots via the xylem, amino acids need to be released into the xylem across the plasma membrane. On the other hand, the higher concentrations of amino acids found in phloem sap compared to the apoplasm of leaf tissues suggests the presence of active transport mechanism to load amino acids to phloem (Lohaus and Heldt, 1995). Furthermore, physiological investigations of amino acid transport in plants have suggested the presence of multiple carriers with broad substrate specificity (Mounoury *et al.*, 1984; Wyse and Komor, 1984).

The first plant amino acid transporter, amino acid permease 1 (AAP1), was identified in *Arabidopsis* by the functional complementation of a yeast mutant strain defective in proline uptake (Frommer *et al.*, 1993). Following this discovery, a number of other plant amino acid transporters were identified by similar functional complementation of yeast mutants. In *Arabidopsis*, at least 53 AAP homologs (also known as NRT1 family) have been found by homology search. (summarized by Wipf *et al.*, 2002). The biochemical analyses conducted on the identified amino acid transporters have suggested that they are multi-substrate transporters and are energized by proton co-transport (Hirner *et al*, 1998; Okumoto *et al*., 2002; 2004). These characterized AAPs are specifically expressed in vascular tissue or in reproductive organs (Hirner *et al*, 1998; Okumoto *et al*., 2002; 2004). The expression patterns of these AAP homologs suggest that they are involved in the translocation of amino acids via the xylem and phloem, and their role may be particularly important during the reproductive growth stage when amino acids (mostly glutamine and asparagine) are transported to developing seeds from senescing leaves (Hirner *et al*, 1998; Okumoto *et al*., 2002). Nevertheless, the mutants for these amino acid transporters do not show any phenotype, possibly due to the functional redundancy among the AAP homologs; therefore the specific functions of each AAP homolog is currently obscure (Hirner *et al*, 1998; Okumoto *et al*., 2002; 2004).

1.3.2. Direct uptake of exogenous amino acids by plant roots

In some terrestrial ecosystems, such as arctic tundra or boreal forests, where the decomposition of organic matter is slow, both inorganic and organic N are present in soils (Lipson and Näsholm, 2001). The organic form of N in such soils is composed of proteinaceous material, heterocyclic compounds (e.g. nucleotides) and amino acids (Schulten and Schnitzer, 1998). In terms of the uptake of amino acids, it is thought that microorganisms outcompete plants for the uptake of amino acids, and plants access only inorganic N that is released in excess of the microbial requirements (Lipson and Näsholm, 2001). However, some plant species, including trees and some species found in arctic tundra, are capable of efficient amino acid uptake from soils (Ohlund and Näsholm, 2001; McKane *et al*., 2002). The evidence of amino acid uptake by plants was provided by a tracer study with a double, universally labeled amino acid, [$^{13}C_2$, ^{15}N] glycine (Näsholm *et al*., 1998). In this experiment, the use of the doubly labeled glycine allowed them to determine if plants were directly taking up glycine or taking up ammonium generated from glycine by microbial activity. The increase in both ^{13}C and ^{15}N content detected in the plant extracts demonstrated the direct uptake of amino acids by plants. However, the extent of plant amino acid uptake depends on plant species, microbial activity, soil types and climate, and it cannot be generalized for plants growing in different ecosystems (Lipson and Näsholm, 2001).

The molecular identity of the amino acid transporter(s) mediating the uptake of amino acids from soils has been recently reported. It was found that LHT1, originally identified as a lysine- and histidine-specific amino acid transporter (Chen and Bush, 1997), was a high affinity amino acid transporter with broad-specificity and was responsible for the amino acid uptake at the root epidermis (Hirner *et al.*, 2006). The involvement of LHT1 in the uptake of externally supplied amino acids was evidenced by the impaired growth of the *lht1* mutants on media containing aspartate as an N source and the slowed rate of labeled amino acid uptake in *lht1* mutant (Hirner *et al.*, 2006). In addition, *lht1* mutants exhibited growth defects when grown with normal inorganic N supply. However, this phenotype was recovered by the tissue-specific expression of a normal *LHT1* gene in green tissues, demonstrating that this phenotype was independent of the function of LHT1 in roots. Analysis of amino acid content in leaves further substantiated the involvement of LHT1 in the amino acid transport across the membrane of mesophyll cells (Hirner *et al.*, 2006). Furthermore, another group has recently shown that LHT1 was responsible for the amino acid uptake in roots (Svennerstam *et al.*, 2007). In their experiments, they employed an unbiased forward genetics screen for mutants defective in amino acid uptake. Since D-Ala is taken up by plants but is highly toxic (Erikson *et al.*, 2004), they screened for the plants resistant to D-Ala. The recovered D-Ala resistant plant was shown to have a mutation in the *LHT1* gene, confirming the results of Hirner *et al.* (2006). Although LHT1 exhibits a wide range of substrate-specificity (Hirner *et al.*, 2006), uptake of several amino acids (i.e. lysine and glutamate) were not affected in *lht1* mutants, suggesting the existence of other transporters mediating amino acid uptake at root surface. (Svennerstam *et al.*, 2007).

1.4. The response of plants to oxygen deficiency

Plants experience oxygen deficiency when subjected to conditions of excess water. Since oxygen limitation is a primary stress having profound effects on plant growth and development, the physiology of plants subjected to low oxygen stress has been extensively studied. Typically, the dramatic shift in metabolism in response to the fall in oxygen concentrations involves not only energy production pathways but also amino acid metabolism. This section covers the strategies employed by plants to cope with this stress. (See Appendix 1 for specific reactions of the related enzymes).

1.4.1. Hypoxic stress

Fully aerated soils receive a constant supply of oxygen by rapid gas exchange between the soil space and ambient air; however, this transfer of oxygen into root environment is blocked when the soil pore space is filled with water (Drew, 1997; Vartapetian and Jackson, 1997). This is due to the significantly slower diffusion of gas (in particular oxygen) in water than in air (10,000 times slower) (Armstrong, 1979). Under conditions of excess water, the soil environment can become devoid of oxygen within 24 hrs since soil microorganisms and plant roots rapidly consume the remaining oxygen (Erdmann and Wiedenroth, 1988; Drew, 1997). Oxygen limitation is thus associated with submergence, transient flooding, waterlogged soils and irrigation practices in the soils of poor drainage.

Excess water in soils can cause a range of oxygen limitation, from a small drop in the oxygen concentration to the complete loss of oxygen. With regard to the terminology, "anoxic" and "anaerobic" are generally used for the complete deprivation of oxygen (i.e. 0 % O_2), while "hypoxic" and "low oxygen conditions" are more loosely used to describe a range of conditions with reduced oxygen levels (< 10 % O_2). Under anoxic conditions, complete oxygen deficiency stops ATP synthesis by mitochondrial oxidative phosphorylation, and the operations of glycolysis and fermentation become the sole energy production pathway (Drew, 1997). On the other hand, mitochondrial oxidative phosphorylation becomes "limited" by the decreased oxygen concentrations under hypoxic conditions (Drew, 1997). The fact that relatively mild oxygen limitation (e.g. 10 % O_2) decreases the rate of respiration suggests that oxygen limitation frequently affects growth and development of plants during their lifecycle (Jackson *et al.*, 1982; Geigenberger, 2003). In the remainder of this thesis, "hypoxic" is used to loosely describe oxygen-limiting conditions, while "low oxygen stress" is used for a range of oxygen deficiency including both hypoxic and anoxic conditions.

1.4.2. Impact of oxygen limitation on plant growth

Oxygen supply is an absolute requirement for the growth of higher plants as it is required for the fundamental metabolic processes in plants, such as respiration and various oxidation, oxygenation reactions (Drew, 1997). In general, higher plants are unable to survive for more than a few days of oxygen deprivation (Drew, 1997). In flooding or waterlogging soils, oxygen limitation is the major cause of the plant growth inhibition. During 1993, approximately 20 million acres of corn and soybean were affected by flooding in the mid-western United States resulting in the large economic loss (Suszkiw, 1994). In Western Australia, 10-15% (up to more than 50%) of wheat yield is estimated to be lost due to waterlogging, resulting in a huge economical loss in the order of 300 million Australian dollars per annum (Dennis *et al.*, 2000).

The detrimental effects of anoxic/hypoxic stress are mostly caused by the inhibition of mitochondrial oxidative phosphorylation and cytosolic acidosis. Prolonged anoxia causes ATP deficiency and severe cytoplasm acidification (Drew, 1997). Cell death caused by anoxia appears to be closely associated with these cellular phenomena (reviewed by Drew, 1997). Acidification of the cytoplasm (pH 7.3 – 7.4 under normoxic conditions) is initially slow and pH is maintained for several hrs at a pH of 6.8 – 7.0 (Drew, 1997; Felle, 2005). The subsequent steep decrease in cellular pH occurs after fermentation-mediated ATP production has stopped due to the depletion of fermentable sugars. This energy deficiency restricts the function of the proton-translocating ATPases in the plasma membrane and the tonoplast, resulting in a pH decrease, since the extrusion of protons from the cytoplasm becomes impaired and protons stored in the vacuoles are released into the cytoplasm by passive leakage (Drew, 1997; Felle, 2005). The consequent decrease in cytosolic pH then triggers cell death in plants. In contrast to the severe effect of anoxic stress, hypoxic stress generally does not result in cell death but inhibits plant growth. Typically, oxidative phosphorylation is slowed at an O_2 concentration far above the K_m (oxygen) of cytochrome oxidase, and the rate of ATP consumption is concomitantly reduced (Geigenberger, 2003). Along with these changes in energy production metabolism, other important processes, such as photosynthesis and nutrient uptake, are affected under hypoxic conditions, resulting in inhibition of plant growth and development. In addition, as internal hypoxic conditions are present throughout the plant (e.g. stele, pistil style, apical meristems), adjustment of the cellular metabolism in response to mild hypoxia may be a strategy to avoid internal anoxia (Geigenberger, 2003). The rapid response of plants to mild hypoxia also points to the existence of a sensing mechanism and the signal transduction pathway activated by the drop in oxygen levels (Geigenberger, 2003; Subbaiah and Sachs, 2003).

1.4.3. Anaerobic polypeptides (ANPs)

In response to hypoxia, plants undergo a dramatic change in gene expression. The *de novo* synthesis of approximately 20 polypeptides (ANPs for anaerobic polypeptides) was initially reported in anoxically treated maize roots (Sachs *et al.*, 1980, Sachs *et al.*, 1996). These proteins expressed under oxygen limitation were subsequently identified as enzymes involved in glycolysis and fermentation pathways (i.e. ethanol and lactate fermentation) (Sachs *et al.*, 1996). Induction of these ANPs activates anaerobic respiration and allows ATP production through the coupled reaction of glycolysis and fermentation (Sachs *et al.*, 1996; Drew, 1997; Vartapetian and Jackson, 1997). Besides the glycolytic and fermentative enzymes, enzymes involved in N metabolism (e.g. AlaAT, NR), cell wall loosening (xyloglucan endotransglycosylase: XET) and ethylene biosynthesis (1-aminocyclopropane-1-carboxylic acid synthase: ACC synthase) were also identified as enzymes induced by low oxygen stress (reviewed by Dennis *et al.*, 2000). Microarray experiments have provided a more complete list of genes whose expression is affected by low-oxygen stress (Klok *et al.*, 2002; Branco-Price *et al.*, 2005; Gozali *et al.*, 2005; Liu *et al.*, 2005; Loreti *et al.*, 2005; Lasanthi-Kudahettige *et al.*, 2007). These microarray experiments not only supported the well-known induction of sucrose metabolism and ethanol fermentation but also indicated that the response to low oxygen stress involves more global rearrangement of diverse metabolic processes (Gozali *et al.*, 2005). Nevertheless, whether the induction at transcript levels is correlated with that at the protein level needs to be carefully investigated in order to define the role of a differentially expressed gene in response to low oxygen stress. In fact, large discrepancies between mRNA and protein abundance were revealed by the comparison of microarray data with the corresponding proteome data in response to salt stress (Jiang *et al.*, 2007). Under low oxygen stress, selective translation of the stress-induced transcripts has been reported (Bailey-Serres, 1999). This has been shown to occur due to the difference in the tendency of transcripts to be associated with the ribosomes under oxygen deprivation (Bailey-Serres and Freeling, 1990; Bailey-Serres and Dawe, 1996).

1.4.4. Structural and developmental response to hypoxia

When subjected to low oxygen stress, plants undergo structural and developmental responses. These responses are often induced by the exposure to hypoxic stress and lead to permanent morphological changes in an attempt to avoid oxygen deficiency. The development of aerenchyma, which is an interconnected gas-filled channel in stems or roots, is a good example of the structural response to falling oxygen levels (Drew, 1997; Vartapetian and Jackson, 1997). Aerenchyma allows oxygen delivery from the aerial parts of plants to the tissues in anaerobic environment (Drew, 1997; Vartapetian and Jackson, 1997). Roots growing in waterlogged soils can be sufficiently oxygenated by the gas exchange through aerenchyma. The formation of aerenchyma in wetland species is generally independent of the environmental stimuli, while that in non-wetland species is only induced by oxygen deprivation. In non-wetland species, aerenchyma formation is linked to cell death of cortical cells triggered by the increase in ethylene biosynthesis under the stress conditions (Vartapetian and Jackson, 1997; Drew *et al.*, 2000). In addition, plants often develop adventitious roots to replace the existing root systems that have been killed or damaged by oxygen deficiency (Vartapetian and Jackson, 1997). Adventitious roots emerge from the shoot base and grow near the surface of flooded soils or water, retaining the access to ambient oxygen.

Besides these responses, plants also enhance shoot elongation when subjected to submergence (i.e. elongation of stem, petiole, leaf, and coleoptile) (reviewed by Vartapetian and Jackson, 1997). This rapid elongation enables a part of shoots to remain above the surface of water, securing the access to oxygen. In deepwater rice, internode elongation is stimulated by the rise of the water surface level during the rain season (Kende *et al.*, 1998). Induced ethylene biosynthesis under submergence and the entrapment of this gaseous hormone by surrounding water increases ethylene concentration at the internode (Kende *et al.*, 1998). This increase in ethylene concentration in turn enhances the sensitivity of cells to the elongation-promoting properties of bioactive gibberellins (GAs), thereby enabling fast internode elongation in water (Kende *et al.*, 1998). In addition, coleoptile elongation of germinating rice seedlings has been extensively studied for its unusual growth capacity under anoxia (Menegus *et al.*, 1993; Vartapetian and Jackson, 1997). The utilization of the stored C in rice seeds (mostly starch) allows a high metabolic state in rice coleoptiles even under anoxic conditions (Vartapetian and Jackson, 1997). Enzymes involved in cell wall loosening, such as XET and expansins, have been suggested to play a role in the rapid cell elongation by allowing the extension of the cell wall. The induction of these enzymes has been demonstrated in both stressed deepwater rice and rice coleoptiles (Vartapetian and Jackson, 1997; Kende *et al.*, 1998; Lasanthi-Kudahettige *et al.*, 2007).

1.4.5. Metabolic response to hypoxia: carbon metabolism

Plants are generally tolerant to a short period of oxygen deficiency (at least for a few hrs). Their tolerance is largely due to the operation of anaerobic metabolism. In response to the fall in oxygen concentrations, the rate of mitochondrial respiration slows or stops, due to the shortage of the terminal electron acceptor (O_2). To compensate for the impairment of the aerobic respiration, plants activate the anaerobic metabolic pathways in order to produce ATP in an oxygen-independent manner. The synthesis of ATP and the regeneration of NAD^+ is made possible by the coupling of glycolysis with fermentation (Drew, 1997; Sousa and Sodek, 2002). Since the ATP production through this process is inefficient compared to the aerobic respiration (2 ATP versus 36 ATP per molecule of glucose), carbohydrate consumption can be increased to meet the demand of ATP under low oxygen conditions. This increase in glycolysis flux is called the "Pasteur effect". Nevertheless, the Pasteur effect does not show any correlation with the tolerance of plants to low oxygen stress (Kennedy *et al.*, 1992; Sousa and Sodek, 2002).

In general, the most important anaerobic metabolism is ethanol fermentation. In this simple pathway, pyruvate, the end product of glycolysis, is converted to acetaldehyde by pyruvate decarboxylase (PDC) and then to ethanol by alcohol dehydrogenase (ADH) (Figure 1.7). The characterization of *adh* and *pdc* mutants in maize and *Arabidopsis* demonstrated the crucial role of ethanol fermentation in the stress tolerance since these mutants rapidly succumbed to oxygen deprivation (Kennedy *et al.*, 1992; Ismond *et al.*, 2003; Kürsteiner *et al.*, 2003). In terms of ethanol production, plants contain excess ADH activity compared to PDC activity; thus, the production of ethanol is generally limited by PDC (Rahman *et al.*, 200; Ismond *et al.*, 2003). After ethanol is produced in the stressed tissues, ethanol rapidly escapes to the extracellular space since this molecule is highly permeable to membrane. Although the extrusion of ethanol results in the loss of C, this prevents the accumulation of ethanol, which is toxic to cell (Sousa and Sodek, 2002).

Another important anaerobic respiration is lactate fermentation, in which pyruvate is converted to lactate by lactate dehydrogenase (LDH) (Figure 1.7). However, C flux into lactate fermentation is usually much smaller than into ethanol fermentation in most species (Fan *et al.*, 1997). Nevertheless, the importance of the lactate production may lie in the control of cytosolic pH and the regulation of ethanol fermentation (Davies *et al.*, 1974; Davies, 1980). It has been suggested that lactate formation is initiated at the onset of low oxygen stress, and its accumulation causes cytosolic acidification. The drop in cytosolic pH then activates PDC that has an acidic pH optimum, but it in turn inhibits LDH activity. In this proposed pH-stat model, lactate production functions as the switch for the shift from lactate to ethanol fermentation when subjected to low oxygen stress (Davies, 1980). However, many studies have argued against the pH-stat hypothesis. For instance, lactate production was not correlated well with the fall in pH, and lactate production continued long after the initial pH drop in response to low oxygen stress (Saint-Ges *et al.*, 1991; Sakano *et al.*, 1997; reviewed by Felle, 2005). Moreover, activity of PDC may not be strictly controlled by pH, thus the sharp pH-dependent switch between lactate and ethanol production is very unlikely (Felle, 2005). Although the pH-dependent switch seems doubtful, a recent study showed that the modulation of LDH activity resulted in the changes in PDC activity, suggesting some extent of interdependency between ethanol and lactate fermentation (Dolferus *et al.*, 2008). In addition, similar to the extrusion of ethanol, plants appear to excrete lactate from roots when lactate is constantly produced under low oxygen stress (Rivoal and Hanson, 1993; Dolferus *et al.*, 2008).

The shift from aerobic respiration to anaerobic fermentation under low oxygen stress is often accompanied with the alteration in sucrose metabolism and the simultaneous acceleration of glycolysis (Geigenberger, 2003; Fukao and Bailey-Serres, 2004). Plants preferentially use the reversible plant-specific sucrose synthase (SUS) pathway to hydrolyze sucrose under the stress conditions since the SUS pathway requires less ATP input than the invertase pathway for sucrose breakdown, and thus the pathway is energetically more efficient (Fukao and Bailey-Serres, 2004; also see Figure 1.7). The activation of SUS and the inactivation of invertase were demonstrated at both mRNA and enzyme activity levels in maize roots (Zeng *et al.*, 1999). The efficient operation of SUS pathway is seemingly a prerequisite for the survival of plants during oxygen deprivation (Bologa *et al.*, 2003; Harada *et al.*, 2005). Furthermore, low oxygen stress induces α-amylase in rice, an enzyme that catalyzes starch hydrolysis, increasing the supply of soluble carbohydrates for fermentation (Loreti *et al.*, 2003, Fukao *et al.*, 2006). As identified as ANPs, simultaneous induction of glycolytic enzymes have been also observed in many studies in response to low oxygen stress (Sachs *et al.*, 1996; Fukao and Bailey-Serres, 2004).

The activation of sucrose breakdown, glycolysis and ethanol and lactate fermentations can result in Pasteur effect under oxygen deficiency. However, the increase in carbohydrate consumption is often observed only during the initial phase of the stress, and both ATP production and consumption is reduced during the prolonged oxygen limitation (Geigenberger *et al.*, 2003; Fukao and Bailey-Serres, 2004). In fact, the repression of the genes encoding enzymes catalyzing oxygen-requiring reactions was observed in rice coleoptiles (Lasanthi-Kudahettige *et al.*, 2007). This might be an energy-saving strategy under low oxygen stress. In support of the essential role of the energy-saving mechanisms during the prolonged stress conditions, it has been shown that the ethylene-response-factor-like gene, *Sub1A*, which controls the submergence tolerance in rice, negatively regulates the carbohydrate consumption, leaf and internode elongation, and chlorophyll degradation (Fukao *et al.*, 2006; Xu *et al.*, 2006). Although the expression of *Sub1A* induced PDC and ADH, it repressed the enzymes associated with starch breakdown, sucrose hydrolysis and cell wall loosening (Fukao *et al.*, 2006). The repression of these processes in *Sub1A*-carrying submergence tolerant rice resulted in reduced leaf and internode elongation as well as the better retainment of soluble carbohydrates and chlorophyll during the submergence stress (Fukao *et al.*, 2006). The ability of *Sub1A* to confer the submergence tolerance substantiates the importance of ethylene-signaled repression of energy consuming processes during oxygen deficiency (Perata and Voesenek, 2007).

The other products of anaerobic C metabolism include succinate and malate (Fan *et al.*, 1988; 1997). These substrates of the TCA cycle are likely generated either by the partial and reverse operation of the TCA cycle or by the aspartate metabolism (Sousa and Sodek, 2002). Although the production of succinate and malate may be linked to the additional generation of ATP in mitochondria, their physiological roles under low oxygen stress remains unclear (Sousa and Sodek, 2002).

1.4.6. Metabolic response to hypoxia: amino acid metabolism

Amino acid metabolism undergoes a drastic change in response to low oxygen stress. The most prominent consequence of this metabolic adaptation is the accumulation of alanine and GABA in the stressed tissues (Streeter and Thompson, 1972; Fan *et al.*, 1997; Reggiani *et al.*, 2000). However, the physiological role of this amino acid accumulation is ambiguous since the regeneration of NAD^+, which is the fundamental requisite for the continuance of glycolysis, is not directly linked to the synthesis of these amino acids (Sousa and Sodek, 2002). In terms of the physiological role of this adaptation, it has been hypothesized that the maintenance of the osmotic potential in the stressed tissues is an important function of amino acid accumulation in order to counteract the rapid fall of cellular carbohydrate levels during low stress (Reggiani *et al.*, 2000, Aurisano *et al.* 1995a). Moreover, the production of alanine and GABA has been suggested as an adaptive mechanism to store the carbon and nitrogen that would otherwise be lost under oxygen deficient conditions (i.e. extrusion of ethanol and lactate as well as the unregulated loss of ammonium to the extra cellular spaces) (Sato *et al.* 2002, Ricoult *et al.* 2005).

Alanine can be considered as the third most important fermentation product synthesized from pyruvate under low oxygen stress. (Sousa and Sodek, 2002). When subjected to the stress, alanine is produced in relatively large amounts and becomes one of the most abundant amino acids in plant roots (Streeter and Thompson, 1972; Good and Muench 1993; Fan *et al.*, 1997; Sousa and Sodek, 2003; Ricoult *et al.*, 2005, 2006). For instance, alanine content could account for 1.2 % of the dry weight in excised rice roots after 24 hrs of anoxic stress (Reggiani *et al*., 1985). This accumulation of alanine under oxygen deficiency also indicates a major alteration in the N metabolism. Although ammonium assimilation (i.e. the GS/GOGAT reaction) continues at a reduced rate under low oxygen stress, aassimilated N mostly ends up in alanine under the stress conditions (Vanlerberghe *et al*., 1991; Reggiani *et al*., 2000). In regards to the synthesis of alanine under hypoxia, it has been suggested that AlaAT is the enzyme responsible for alanine production based on its induction profiles in response to low oxygen stress (Good and Crosby, 1989; Muench and Good, 1994; Ricoult *et al*., 2005). However, Sousa and Sodek (2003) have pointed out that most of the increase in AlaAT activity took place after alanine production ceased, suggesting that AlaAT plays a role in alanine catabolism during the recovery from low oxygen stress. While the purpose of hypoxic alanine production remains unclear, evidence suggests that it is important in the survival and nitrogen metabolism of plants under hypoxic conditions. In the single cell alga, *Selenastrum minutum*, approximately 90% of the ammonium assimilated under anaerobic conditions was incorporated into alanine (Vanlerberghe *et al*., 1991). Thus, alanine production under oxygen deficiency is a mechanism by which the alga increases its nitrogen pool in preparation for the return to normoxia. Supporting these findings is the observation that the anaerobic tolerance of rice coleoptile depends upon the assimilation of inorganic nitrogen into alanine and other amino acids (Fan *et al*., 1997). In the legume, *Medicago truncatula*, an inhibitor of GOGAT (azaserine), which was believed to block alanine synthesis by inhibiting glutamate formation, impaired germination and seedling development under anoxic conditions (Ricoult *et al*., 2005).

GABA is another amino acid accumulated in the hypoxically stressed plants (Streeter and Thompson, 1972; Fan *et al*., 1997). Normally, GABA is synthesized in the reaction catalyzed by GAD (Figure 1.6). As this reaction consumes a proton, GABA synthesis has been thought particularly important to alleviate the cytosolic acidification during low oxygen stress (Streeter and Thompson, 1972; Bown and Shelp 1997). In support for this idea, GAD activity is stimulated by acidic conditions and by the increase in Ca_2^+ concentration that leads to the activation of GAD through the interaction with Ca_2^+/calmodulin (Bown and Shelp 1997; Shelp *et al*. 1999). In addition, GABA may play a role in the synthesis of alanine, since the GABA-T reaction, the second step of the

GABA shunt, produces alanine and SSA from pyruvate and GABA (Figure 1.6). This poses the potential of alanine production through the operation of the GABA shunt. Streeter and Thompson (1972) investigated whether the GABA-T reaction contributes to the accumulation of alanine but concluded that this reaction is inhibited under anaerobic conditions and does not play a role in the hypoxia-induced alanine accumulation. They reported no detectable levels of SSA, a slowed rate of [^{14}C]GABA metabolism and the decrease in GABA-T activity under anaerobic conditions as the supporting evidences for their conclusion (Streeter and Thompson 1972). However, it has been recently demonstrated that SSA can be reduced to γ-hydroxybutyrate (GHB) by γ-hydroxybutyrate dehydrogenase (GHBDH, Figure 1.6), and the accumulation of GHB in anaerobically stressed plant tissues was observed (Breitkreuz *et al*. 2003, Allan *et al*. 2003). These results suggest that GABA-T is operative under oxygen deficiency and may contribute to the hypoxia-induced alanine accumulation.

1.5. The response of plants to carbon starvation

C fixation through photosynthesis is the fundamental reaction that provides principally all C sources in plants. Since the N assimilation into amino acids requires 2-oxo-acids, C and N metabolisms are closely coordinated though the interdependent regulation of photosynthesis, respiration and N assimilation (Stitt and Krapp, 1999). Therefore, the availability of C metabolites (sugars, carbohydrates) is a key component of the signal that triggers various changes in plant metabolism, growth and development. In particular, the effect of C starvation on amino acid metabolism is discussed in this section.

1.5.1. Carbon starvation

Although plants acquire carbon by photosynthesis and are generally seen as autotrophs, plants can be considered as C heterotrophs during some part of their life cycle and in non-photosynthetic tissues (Yu, 1999). Since C metabolites in plants are derived from photosynthesis, perturbation of this essential process can lead to the development of C starvation. In fact, photosynthesis can be affected by various biotic and abiotic stimuli, such as pathogen attack, herbivore ingestion, light intensity, water supply and temperature. Under the adverse environmental conditions, heterotrophic cells can become

C limited since the reduction in the photosynthesis rate can result in the decrease in the import of sugars from source tissues (i.e. photosynthesizing leaves) to sink tissues (non-photosynthetic tissues) (Yu, 1999). In addition, it should be noted that prolonged low oxygen stress could cause C starvation during post-stress periods. During low oxygen stress, anaerobic fermentation processes may consume and deplete the soluble sugars. Photosynthesis is also affected during this stress since the stress induces ABA biosynthesis and this hormone triggers stomatal closure, inhibiting the CO_2 acquisition and transpiration (Vartapetian and Jackson, 1997). In addition, photosynthesis can be turned off or its rate can be reduced at the certain stage of plant life cycle, such as annual resting season and after leaf shedding (Yu, 1999). During these periods, plants become reliant on the carbohydrates reserves and may experience C starvation in the C heterotrophic cells.

When carbohydrates become limited, plant cells initiate the sequential changes in diverse metabolic processes (Yu, 1999). First, the depletion of soluble sugars results in a decline in the rate of respiration. This triggers the degradation of lipids and proteins, resulting in the accumulation of inorganic phosphate ions and free amino acids. Moreover, sugar starvation causes the decreases in the enzymes involved in glycolysis, mitochondrial respiration, nitrate reduction and assimilation as well as the concomitant increase in the enzymes related to catabolism of fatty acids, amino acids, and proteins (Yu, 1999). These metabolic changes demonstrate the shift in C metabolism to recycle cellular components during C deficiency. Interestingly, a similar metabolic change also occurs during senescence (Dieuaide *et al.*, 1992; Yu, 1999). In senescing leaves, cellular components are first degraded by autophagy, a process of non-selective bulk degradation of cellular components, in order to recycle respiration substrates (Aubert *et al.*, 1996).

1.5.2. Role of glutamate dehydrogenase under carbon starvation

It is now generally accepted that GDH catalyzes the deamination of glutamate and plays a negligible role in ammonium assimilation (Dubois *et al.*, 2003). In accordance with this proposed direction of the GDH reaction, GDH has been suggested to play a role in the generation of respiration substrate (i.e. 2-oxoglutarate) from glutamate under carbohydrate deficiency (Robinson *et al.*, 1992; Aubert *et al.*, 2001; Miflin and Habash, 2002). Supporting this hypothesis, it has been shown that expression of GDH is closely regulated by the cellular C status. Generally, GDH is induced in the dark or under C starvation conditions, and this induction is repressed by the supply of exogenous sucrose (Robinson *et al.*, 1992; Melo-Oliveira *et al.*, 1996; Masclaux-Daubresse *et al.*, 2002; Restivo, 2004). In this context, it is possible that the induction of GDH promotes the glutamate breakdown to replenish the TCA cycle. Under sugar starvation, the levels of free amino acids increase due to the autophagic recycling of cellular components as discussed above (Yu, 1999). The utilization of the resulting free amino acids as respiration substrates is an important metabolic adaptation to C deficiency, and GDH has been suggested to be involved in this process (Robinson *et al.*, 1992; Masclaux-Daubresse *et al.*, 2002). In addition, the induction of GDH during senescence has been reported (Thomas, 1978; Kar and Feierabend, 1984; Srivastava and Singh, 1987; Masclaux *et al.*, 2000; Masclaux-Daubresse *et al.*, 2002). Whether GDH is induced by the senescence-related sugar starvation or by ammonium accumulation is not clear, however GDH in senescing leaves may function to breakdown glutamate under C deficient conditions.

1.6. Goal of this thesis

Although plant N metabolism has been extensively studied to date, uncertainty remains with regard to the roles played by several N metabolites and the related enzymes. These include the role of alanine and the *in vivo* role of GDH in plant N metabolism. In terms of alanine, the most dramatic change in its metabolism occurs during low oxygen stress, in which alanine is rapidly accumulated in the stressed tissues (Reggiani and Bertani, 2003). On the other hand, GDH has proposed to be a stress-related enzyme, and it may participate in the energy production during C starvation (Dubois *et al.*, 2003). Therefore, this thesis research was targeted to study the mechanism and role of alanine accumulation under hypoxia and the amino acid catabolism during C starvation. Using *Arabidopsis* as a model system. The specific objectives of this study were to;

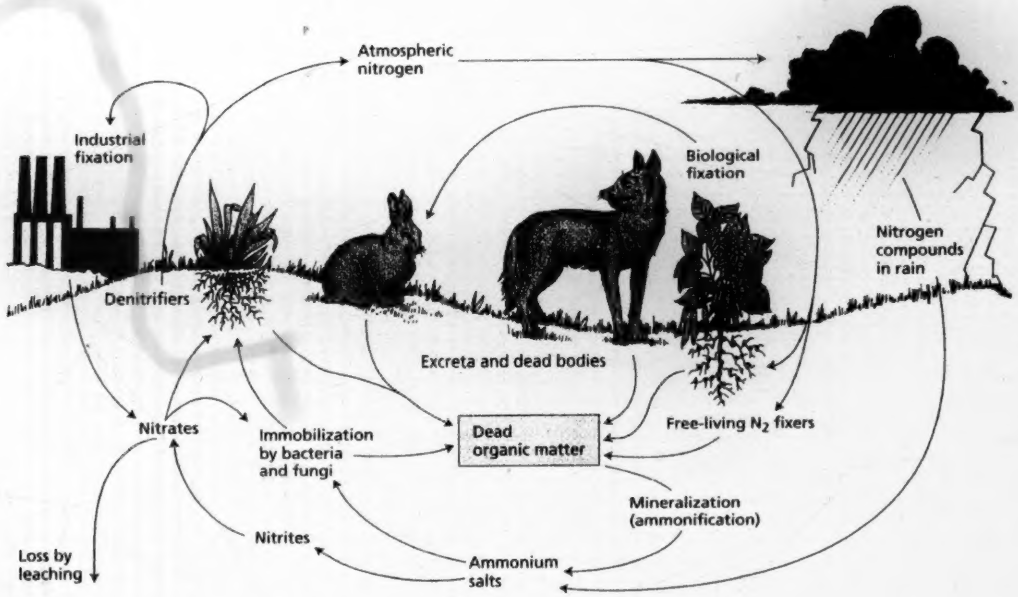
1. Characterize the role of AlaAT under hypoxic conditions through monitoring gene expression and by analyzing T-DNA (*alaat1-1*), TILLING (*alaat2-1* and *alaat2-2*) mutants and over-expressing lines (carrying barley *AlaAT*).

2. Investigate the potential involvement of the GABA shunt in the hypoxic alanine accumulation by monitoring gene expression and by characterizing T-DNA mutants for the GABA shunt enzymes *(gaba-t1-1, pop2-1, pop2-3, gad1-1, gad1-2* and *gad1-3*).
3. Investigate the role of GDH during dark-induced C starvation by monitoring gene expression and by characterizing T-DNA mutants (*gdh1-2, gdh1-3* and *gdh2-1*) and the obtained double knockout mutant (*gdh1-2/gdh2-1*).

Table 1.1. The major processes of biogeochemical nitrogen cycle.

Modified from Taiz and Zeiger (1998).

Process	Definition
Industrial fixation	Industrial conversion of molecular nitrogen to ammonia
Atmospheric fixation	Lightning and photochemical conversion of molecular nitrogen to nitrate
Biological fixation	Prokaryotic conversion of molecular nitrogen to ammonia
Plant acquisition	Plant absorption and assimilation of ammonium or nitrate
Immobilization	Microbial absorption and assimilation of ammonium or nitrate
Ammonification	Bacterial and fungal catabolism of soil organic matter to ammonium
Nitrification	Bacterial (*Nitrosomonas sp.*) oxidation of ammonium to nitrite and subsequent bacterial (*Nitrobacter sp.*) oxidation of nitrite to nitrate
Mineralization	Bacterial and fungal catabolism of soil organic matter to mineral nitrogen through ammonification or nitrification
Volatilization	Physical loss of gaseous ammonia to the atmosphere
Ammonium fixation	Physical imbedding of ammonium into soil particles
Denitrification	Bacterial conversion of nitrate to nitrous oxide and molecular nitrogen
Nitrate leaching	Physical flow of nitrate dissolved in ground water out of the topsoil and eventually into the oceans



Atmospheric nitrogen
Industrial fixation
Biological fixation
Nitrogen compounds in rain
Denitrifiers
Excreta and dead bodies
Nitrates
Immobilization by bacteria and fungi
Dead organic matter
Free-living N_2 fixers
Mineralization (ammonification)
Nitrites
Loss by leaching
Ammonium salts

Figure 1.1. Schematic representation of biogeochemical nitrogen cycle.

Nitrogen cycles through the atmosphere as it changes from gaseous form to reduced ions before being incorporated into organic compounds in living organisms. Some of the steps involved in the nitrogen cycle are shown. Taken from Taiz and Zeiger (1998).

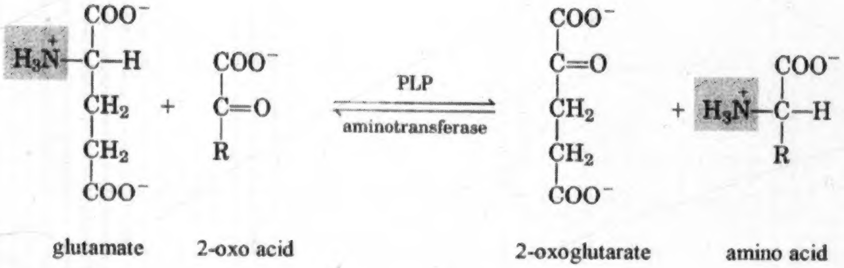

PLP
aminotransferase
glutamate
2-oxo acid
2-oxoglutarate
amino acid

Figure 1.2. A reaction catalyzed by an aminotransferase.

Typical aminotransferases use glutamate as an amino group donor and a 2-oxo acid as an amino group acceptor. Aminotransferase reactions are generally reversible. All aminotransferases require pyridoxal-5-phosphate (PLP) as a cofactor.

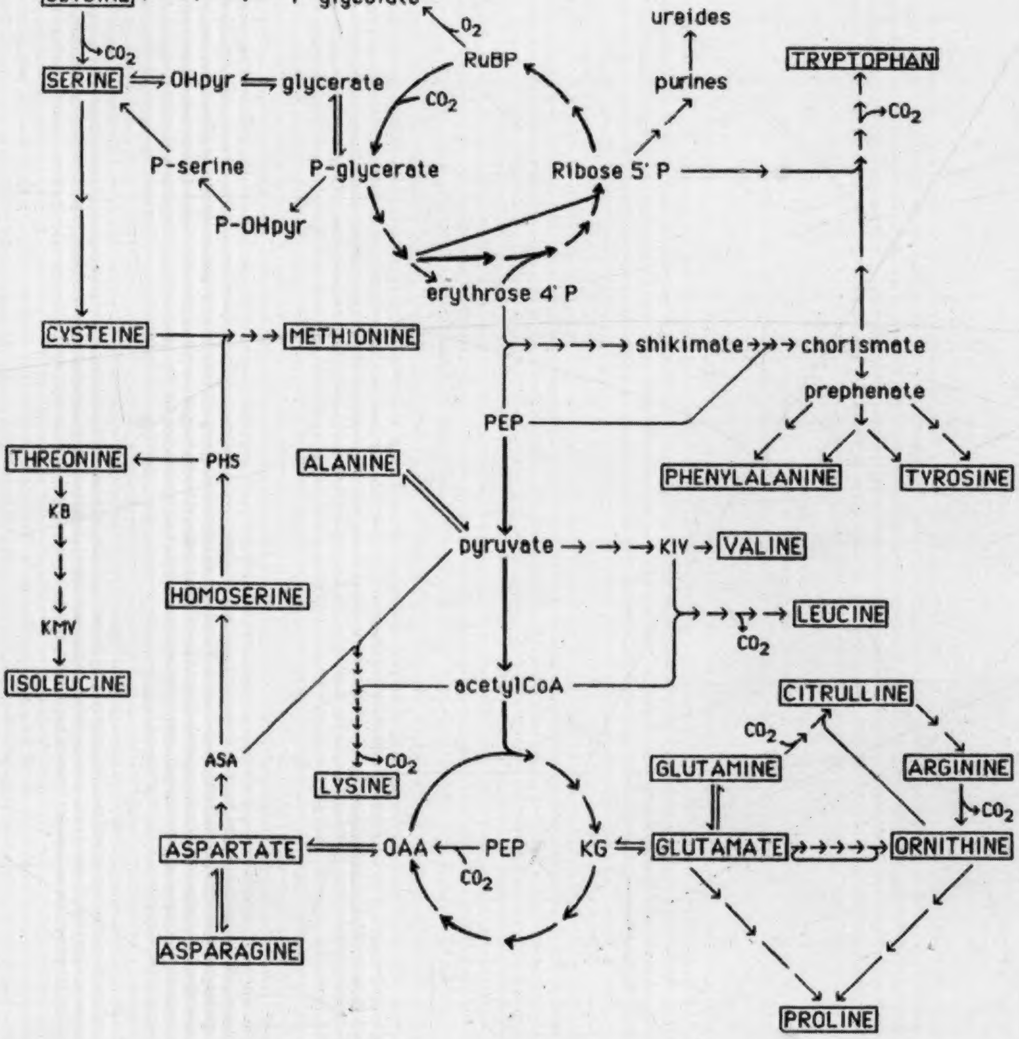

GLYCINE
P-glycolate
O_2
RuBP
ureides
purines
TRYPTOPHAN
CO_2
SERINE
OHpyr
glycerate
CO_2
CO_2
P-serine
P-glycerate
Ribose 5' P
P-OHpyr
erythrose 4' P
CYSTEINE
METHIONINE
shikimate
chorismate
prephenate
PEP
THREONINE
PHS
ALANINE
PHENYLALANINE
TYROSINE
KB
pyruvate
KIV
VALINE
HOMOSERINE
LEUCINE
KMV
CO_2
ISOLEUCINE
acetylCoA
CITRULLINE
CO_2
ASA
CO_2
LYSINE
GLUTAMINE
ARGININE
CO_2
ASPARTATE
OAA
PEP
CO_2
KG
GLUTAMATE
ORNITHINE
ASPARAGINE
PROLINE

Figure 1.3. Overview of amino acid biosynthesis.

The diagram illustrates the carbon flow in the amino acid biosynthesis in plants. Each arrow represents a separate reaction. KIV: ketoisovalerate;, KB: ketobutyrate, KMV: ketomethylvalerate, ASA: aspartate semialdehyde. PEP: phosphoenol pyruvate. Taken from Lea and Ireland (1999).

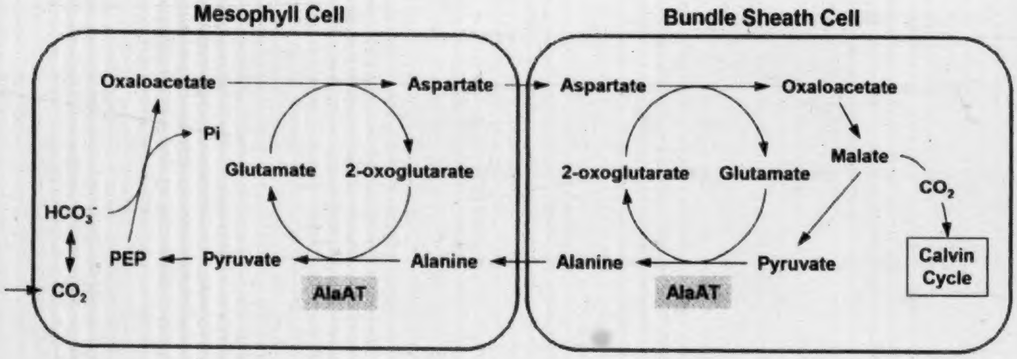

Mesophyll Cell
Bundle Sheath Cell
Oxaloacetate
Aspartate
Pi
Glutamate
2-oxoglutarate
HCO$_3^-$
PEP
Pyruvate
Alanine
CO$_2$
AlaAT
Aspartate
Oxaloacetate
Malate
2-oxoglutarate
Glutamate
CO$_2$
Alanine
Pyruvate
Calvin Cycle
AlaAT

Figure 1.4. Role of AlaAT in C_4 photosynthesis (NAD-malic enzyme type).

The diagram represents the pathway for the C_4 photosynthesis in those plant species that utilize the NAD-malic enzyme to catalyze the decarboxylation of malate. AlaAT is involved in the synthesis of alanine in the bundle sheath cells as well as the conversion of alanine to pyruvate in the mesophyll cells. PEP: phosphoenol pyruvate.

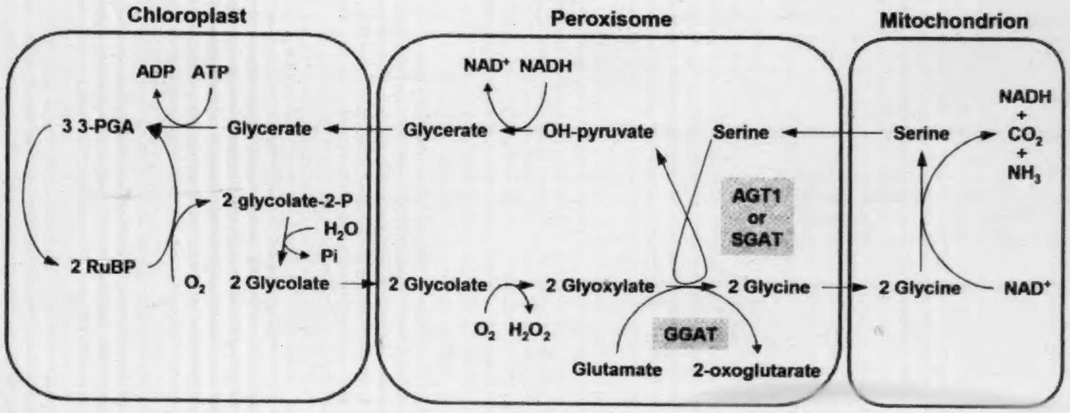

Chloroplast
Peroxisome
Mitochondrion
ADP ATP
3 3-PGA
Glycerate
2 glycolate-2-P
H2O
Pi
2 RuBP
O2
2 Glycolate
NAD+ NADH
Glycerate
OH-pyruvate
Serine
AGT1 or SGAT
2 Glycolate
2 Glyoxylate
2 Glycine
O2 H2O2
GGAT
Glutamate
2-oxoglutarate
Serine
NADH + CO2 + NH3
2 Glycine
NAD+

Figure 1.5. Involvement of aminotransferases in photorespiration.

The diagram depicts the photorespiration pathway. Aminotransferases localized in peroxisomes play important roles in formation of glycine as well as the conversion of serine to hydroxypyruvate. GGAT: glutamate:glyoxylate aminotransferase. AGT: alanine:glyoxylate transaminase, SGAT: serine:glyoxylate aminotransferase. Arabidopsis *AGT1* has been shown to catalyze AGT, SGAT and SPT (serine:pyruvate transaminase) reactions (Liepman and Olsen, 2001; 2003).

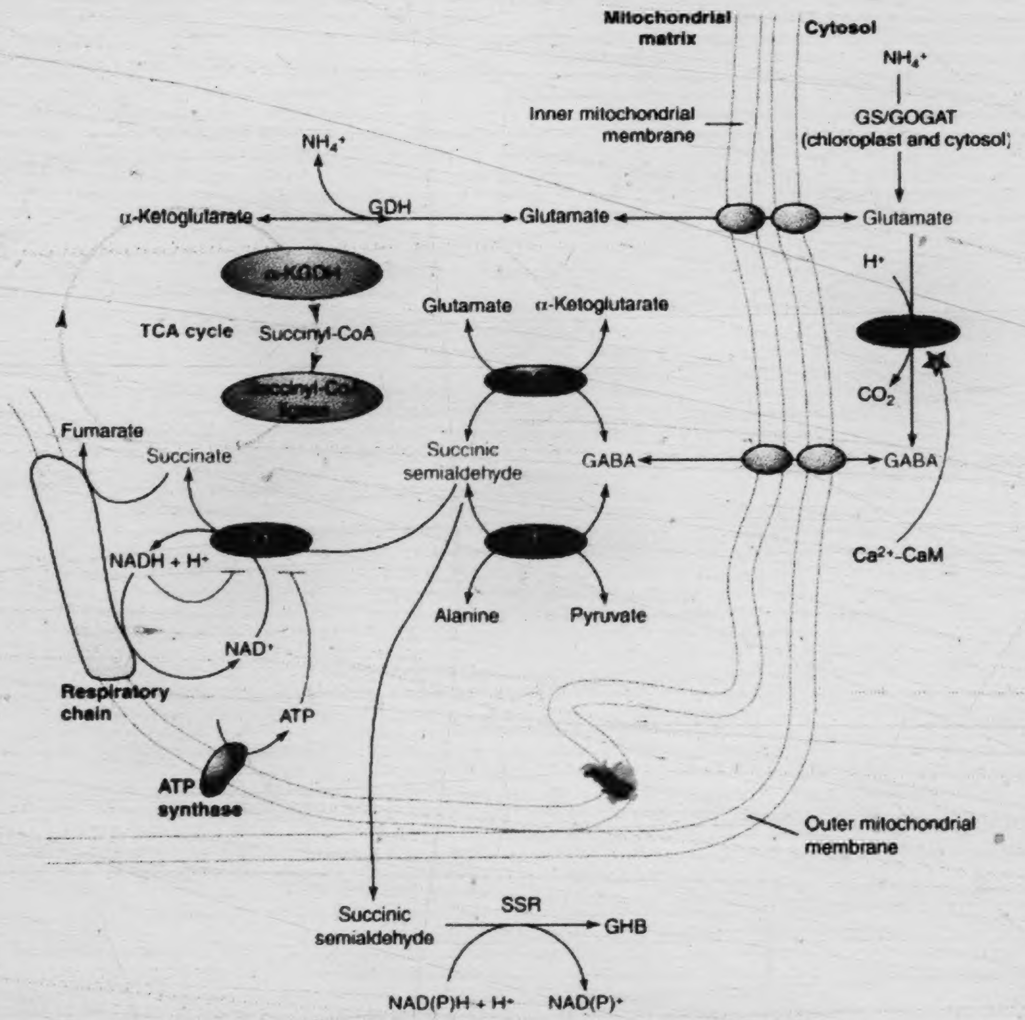

Mitochondrial matrix
Cytosol
NH$_4^+$
Inner mitochondrial membrane
GS/GOGAT (chloroplast and cytosol)
NH$_4^+$
α-Ketoglutarate
GDH
Glutamate
Glutamate
α-KGDH
TCA cycle
Succinyl-CoA
Succinyl-CoA ligase
Glutamate
α-Ketoglutarate
H$^+$
CO$_2$
Fumarate
Succinate
Succinic semialdehyde
GABA
GABA
NADH + H$^+$
NAD$^+$
Alanine
Pyruvate
Ca^{2+}-CaM
Respiratory chain
ATP
ATP synthase
Outer mitochondrial membrane
Succinic semialdehyde
SSR
GHB
NAD(P)H + H$^+$
NAD(P)$^+$

Figure. 1.6. The GABA shunt pathway and its regulation in plants.

The GABA shunt is composed of three enzymes (purple). Glutamate decarboxylase (GAD) converts glutamate to GABA, and its activity is induced by the association with Ca^{2+}/calmodulin (CaM) complex. GABA is converted to succinic semialdehyde in mitochondria by GABA transaminases using either α-ketoglutarate (GABA-TK) or pyruvate (GABA-TP) as amino acceptors. Succinic semialdehyde is then reduced by succinic semialdehyde dehydrogenase (SSADH) to succinate, which enters the TCA cycle. SSADH activity is inhibited by both ATP and NADH levels (green). The GABA shunt bypasses the two enzyme steps (pink), succinyl-CoA ligase and the α-ketoglutarate dehydrogenase (α-KGDH) of the TCA cycle. Succinic semialdehyde can be alternatively converted to γ-hydroxybutyrate (GHB) via a succinic semialdehyde reductase (SSR, also called γ-hydroxybutyrate dehydrogenase: GHBDH). Taken from Bouché and Fromm (2004).

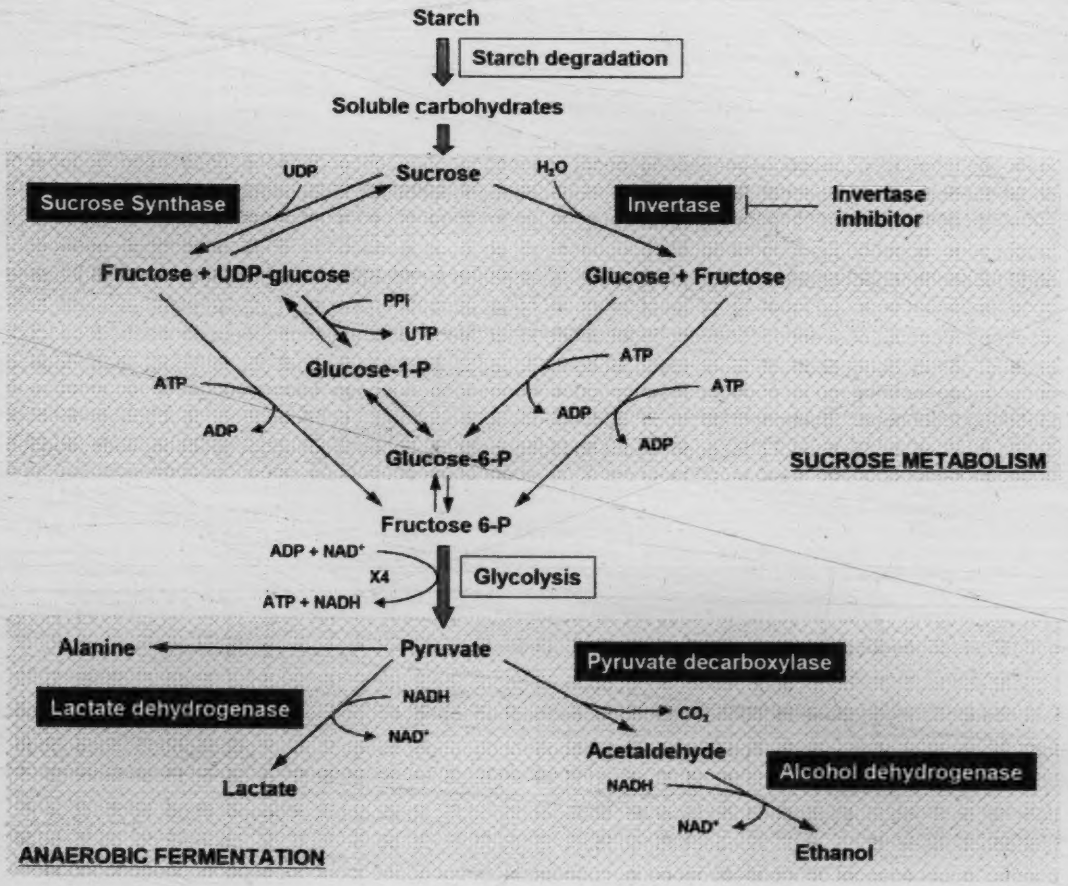

Starch
Starch degradation
Soluble carbohydrates
Sucrose
UDP
H_2O
Sucrose Synthase
Invertase
Invertase inhibitor
Fructose + UDP-glucose
Glucose + Fructose
PPi
UTP
Glucose-1-P
ATP
ADP
ATP
ADP
ATP
ADP
Glucose-6-P
SUCROSE METABOLISM
Fructose 6-P
ADP + NAD⁺
X4
ATP + NADH
Glycolysis
Alanine
Pyruvate
Pyruvate decarboxylase
Lactate dehydrogenase
NADH
NAD⁺
CO_2
Acetaldehyde
Alcohol dehydrogenase
NADH
NAD⁺
Lactate
Ethanol
ANAEROBIC FERMENTATION

Figure 1.7. Pathways of carbohydrate metabolism under low oxygen stress.

Plants have two pathways for the degradation of sucrose. The sucrose synthase pathway is favored under low oxygen stress as this pathway is more energetically efficient than the invertase pathway. In the subsequent fermentation pathways, pyruvate is converted to lactate, ethanol and alanine. The lactate and ethanol formation regenerate NAD^+, while alanine production does not directly regenerate NAD^+. Modified from Fukao and Bailey-Serres (2004).

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Appendix 1. Reactions catalyzed by specific enzymes discussed in Chapter 1.

- **Nitrate reductase** (NR)
  $NO_3^- + NAD(P)H + H^+ + 2\ e^- \rightarrow NO_2^- + NAD(P)^+ + H_2O$

- **Nitrite reductase** (NiR)
  $NO_2^- + 6\ Fd_{red} + 8\ H^+ + 6\ e^- \rightarrow NH_4^+ + 6\ Fd_{ox} + 2\ H_2O$

- **Glutamine synthetase** (GS)
  Glutamate + NH_4^+ + ATP → glutamine + ADP + Pi

- **Glutamate synthase** (NADH-GOGAT and Fd-GOGAT)
  Glutamine + 2-Oxoglutarate + NADH + H^+ → 2 Glutamate + NAD^+
  Glutamine + 2-Oxoglutarate + Fd_{red} → 2 Glutamate + Fd_{ox}

- **Glutamate dehydrogenase** (GDH)
  Glutamate + H_2O + $NAD(P)^+$ ↔ 2-Oxoglutarate + NH_4^+ + NAD(P)H

- **Aspartate aminotransferase** (AAT or AspAT)
  Glutamate + Oxaloacetate ↔ 2-Oxoglutarate + Aspartate

- **Asparagine synthetase** (AS)
  Glutamine + Aspartate + ATP → Asparagine + Glutamate + AMP + PPi

- **Alanine aminotransferase** (AlaAT)
  Glutamate + Pyruvate ↔ 2-Oxoglutarate + Alanine

- **Glutamate:glyoxylate aminotransferase** (GGAT or GGT)
  Glutamate + Glyoxylate ↔ 2-Oxoglutarate + Glycine

- **Glutamate decarboxylase** (GAD)
  Glutamic acid (or Glutamate + H^+) → GABA + $CO_2\uparrow$

- **GABA transaminase** (GABA-T)
  GABA + Pyruvate ↔ Succinic semialdehyde + Alanine
  GABA + 2-Oxoglutarate ↔ Succinic semialdehyde + Glutamate

- **Succinic semialdehyde dehydrogenase** (SSADH)
  Succinic semialdehyde + H_2O + $NAD(P)^+$ → Succinate + NAD(P)H + H^+

- **γ-hydoxybutyrate dehydrogenase** (GHBDH; or succinic semialdehyde reductase, SSR)
  Sccinic semialdehyde + NAD(P)H + H^+ ↔ γ-Hydoxybutyrate + $NAD(P)^+$

- **Pyruvate decarboxylase** (PDC)
  Pyruvic acid (or Pyruvate + H^+) → Acetaldehyde + $CO_2\uparrow$

- **Alcohol dehydrogenase** (ADH)
  Acetaldehyde + NADH + H^+ → Ethanol + NAD^+

- **Lactate dehydrogenase** (LDH)
  Pyruvate + NADH + H^+ → Lactate + NAD^+

2. Alanine aminotransferase catalyzes the breakdown of alanine after hypoxia in *Arabidopsis thaliana*.

2.1. Introduction

Transient flooding often causes oxygen deprivation in the rhizosphere. Such oxygen limitation adversely affects plant performance, as plant roots require sufficient oxygen supply to attain their full functions. Plants respond to low oxygen stress by activating the pathway of anaerobic metabolism. The *de novo* synthesis of approximately 20 anaerobic polypeptides (ANPs) (Sachs *et al*., 1980) triggers the metabolic shift from oxidative phosphorylation to anaerobic fermentation. These ANPs, including the enzymes of glycolysis and anaerobic fermentation pathways, allow limited amounts of ATP to be synthesized in an oxygen independent manner, typically resulting in the production of ethanol and lactate (Sachs *et al*., 1996; Vartapetian and Jackson, 1997). In addition, the parallel expression of genes encoding enzymes involved in nitrogen assimilation and amino acid metabolism also indicates that diverse metabolic pathways operate under low oxygen conditions (Dennis *et al*, 2000; Liao and Lin 2001). The production and accumulation of certain amino acids (i.e. alanine and GABA) is indeed another common response of plants subjected to low oxygen stress (Streeter and Thompson, 1972; Vanlerberghe *et al*, 1991; Ricard *et al*., 199[illegible]; Reggiani and Bertani, 2003).

Alanine aminotransferase (AlaAT; E.C. 2.6.1.2) converts pyruvate and glutamate to alanine and 2-oxoglutarate. In doing so, it is involved in both carbon and nitrogen metabolism in plants. *Arabidopsis* contains four AlaAT homologs. Two of these homologs encode GGAT1 and GGAT2, which have both AlaAT activity and glutamate:glyoxylate aminotransferase (GGAT) activity (Igarashi *et al.*, 2003; Liepman and Olsen, 2003). The presence of a peroxisomal targeting signal in both GGAT1 and GGAT2 suggests that they are localized in the peroxisomes (Liepman & Olsen, 2003), and subcellular fractionation analysis has found GGAT activity to be localized

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exclusively within *Arabidopsis* peroxisomes (Igarashi *et al.*, 2003; Liepman and Olsen, 2003). GGATs appear to play important roles in photorespiration and amino acid metabolism (Liepman and Olsen, 2003, Igarashi *et al.*, 2003). The other two genes, *AlaAT1* and *AlaAT2*, have not been characterized in detail. *In silico* predictions suggest that AlaAT2 is localized in mitochondria while AlaAT1 is in cytosol (Liepman and Olsen, 2003). In fact, the cytosolic localization of a GFP-AlaAT1 fusion protein was observed in tobacco BY-2 cells (Igarashi *et al.*, 2003).

The induction of *AlaAT* during hypoxia has been reported for several plant species including barley (Good and Crosby, 1989; Muench and Good, 1994), maize (Muench *et al.*, 1998), and soybean (Sousa and Sodek, 2003). Recent microarray studies have also demonstrated up-regulation of *AlaAT1* in *Arabidopsis* during hypoxia (Klok *et al.*, 2002; Loreti *et al.*, 2005). Furthermore, induction of *AlaAT* by other stresses has been observed; for example, *AlaAT* expression is regulated by light and nitrogen stress in leaves of *Panicum miliaceum* (Son *et al.*, 1991, 1992). *AlaAT* was also up-regulated during recovery from nitrogen stress in maize (Muench *et al.*, 1998). It is not unusual for enzymes to be involved in more than one stress response. Alcohol dehydrogenase (ADH) is likewise induced by several stresses including wounding, low oxygen, cold, ABA and heat (Dolferus *et al.*, 1994a).

AlaAT expression has been detected in several different tissues and organs. In addition to *AlaAT* expression in roots and leaf tissues, which is observed in most plant species, *AlaAT* expression has been detected in the inner endosperm tissues of developing rice seeds (Kikuchi *et al.*, 1999) and floral organs in *Arabidopsis* (Igarashi *et al.*, 2003). The versatile expression profile of *AlaAT* suggests the involvement of this enzyme in various physiological processes throughout the life cycle of plants.

In this study, An *AlaAT1* T-DNA knockout mutant was also analyzed to characterize the role of this enzyme in hypoxic alanine production and plant survival during low oxygen stress. Putative *AlaAT2* mutants identified by TILLING were also used in this study. In addition, Barley *AlaAT* was overexpressed to assess the potential benefits of altering *AlaAT* expression under low oxygen conditions. The results suggested that the alanine accumulation under hypoxia did not require AlaAT activity; but indicated that AlaAT activity contributes to the rapid conversion of accumulated alanine to pyruvate during post-hypoxia. Moreover, the tissue specific expressions of both *AlaAT1* and *AlaAT2* were investigated using promoter:*GUS* fusions. Based on the results obtained through these experiments, the physiological role of AlaAT in *Arabidopsis* is discussed.

2.2. Materials and Methods

2.2.1. Plant growth conditions and hypoxic stress treatment

Arabidopsis thaliana ecotypes Col-0 and C24 were used. Seeds were sterilized and sown on nylon mesh circles that were placed on top of 0.5x MS medium containing 1% sucrose and 0.4% agar; unless otherwise indicated. Plants were grown under similar conditions as outlined in Ellis *et al.* (1999). Plants were grown on nylon mesh plates for 10 days. Then, the plants grown on the nylon mesh were transferred from solid media to liquid 0.5x MS media containing 1% sucrose and were grown with gentle agitation on a rotary shaker for an additional 7 days. Tissues harvested from these 17-day-old plants were used for all RNA, protein and metabolite experiments.

For the low oxygen stress treatment, 16-17-day-old plants grown as described above were transferred to a new pétri dish containing 10 ml liquid 0.5x MS media (1% sucrose) that had been first gassed for 15 min with 5% oxygen (balance nitrogen). Timentin was added to the liquid media (25mg/l) to prevent bacterial contamination. For each pétri dish, four half-cut microfuge tubes were placed inside to slightly lift the lids of the plates and to allow the gas exchange during hypoxic treatment. These plates were then stacked in anaerobic jars (Ismond *et al.* 2003). The anaerobic jars were flushed with 5% oxygen for an additional 15 min and left in the dark throughout the duration of the treatments. Control experiments were performed by transferring the plants to a new pétri dish containing aerobic liquid 0.5x MS media with addition of timentin (25mg/l). These plates were placed on an orbital shaker and kept in the dark for 24 h.

2.2.2. RT-PCR analysis

Total RNA was isolated from roots and shoots of 17-day-old plants using an RNeasy Plant Mini Kit (Qiagen; http://www.qiagen.com/). The isolated total RNA was treated with DNaseI (DNA-free®, Ambion; http://www.ambion.com/), and first-strand cDNA was synthesized using an oligo $dT_{12\text{-}18}$ primer (Invitrogen;

http://www.invitrogen.com/) and reverse transcriptase, Superscript II (Invitrogen). PCR was performed using the synthesized first strand cDNA with the following gene specific primers; for *AlaAT1* (5'-CTACGGTGAGAAGGATCTTG-3' and 5'-TGCTCTCACATCTTAGCTTCTG-3'), *AlaAT2* (5'-GGGCTATGGAGAAAAAGATATCT-3' and 5'-AGTTCATCTTCATGGTCAGGTC), *GGAT1* (see Liepman and Olsen, 2003), *GGAT2* (5'-GAGTTCGGAAAGAAGTCGCTGAG-3' and 5'-GAGTTTGACACAGAGTAGGACCAG-3'), barley *AlaAT* (5'-ACCTGATGCATTCTATGCTCTT-3' and 5'-GGTTGTATGTAATCCCACATGTT-3'), *Actin2* (5'-CACTATGTTCTCAGGTATCGCT-3' and 5'-TTTTAAGCCTTTGATCTTGAGAGC) and *β-Tubulin* (see Kang and Singh, 2000).

2.2.3. Analysis for tissue specific expression of *AlaAT* transcripts.

To generate promoter:*GUS* fusion constructs, approximately 1000-bp upstream regions of *AlaAT1* and *AlaAT2* were amplified from Col-0 genomic DNA using Platinum Taq DNA polymerase High Fidelity (Invitrogen) and cloned into pCR2.1-TOPO (Invitrogen) by TA-cloning with the following primer sets; for the *AlaAT1* promoter (5'-CCTCACTGCAAAACAGCAACA-3' and 5'-TTGTGATTGTGTGGTATGGGA-3') and for the *AlaAT2* promoter (5'-GCTTCGAAGAACTATTAGTCCCAA-3' and 5'-CTGTGAATCAGCTTTATACTAGGAAC-3'). The inserts were sequenced to confirm the cloning of the correct promoter regions. To introduce EcoRI and NcoI sites at the ends of the promoter fragments, the cloned promoter regions were amplified by PCR with the following primers containing the desired restriction enzyme recognition sequences; EcoRI- *AlaAT1* (5'-AACCGGAATTCCCTCACTGCAAAACAGC-3'), NcoI-*AlaAT1* (5'-TCTTGACCATGGGTGATTGTGTGGTATGG-3'), EcoRI-*AlaAT2* (5'-AACCGGAATTCGCTTCGAAGAACTATTA-3'), NcoI-*AlaAT2* (5'-TCCTGACCATGGGTGAATCAGCTTTATACT-3'). The amplified products were cut with the corresponding restriction enzymes and inserted into pCAMBIA1305.1. The constructs were then introduced into *Arabidopsis* plants (ecotype Col-0) using a modified *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). Transformed plants were selected based on hygromycin resistance and subsets of T3 homozygous lines that displayed observable GUS expression patterns were then selected.

The expression of both *AlaAT* promoters was examined at different developmental stages under aerobic and 24 h hypoxic conditions. Plants were grown on vertical 0.5x MS plates (1% sucrose, 1% agar) for 9-20 days. Plants were then hypoxically treated (5% oxygen) for 24 h in the dark, while control plants were left in air for 24 h in the dark. *In vivo* GUS staining was performed as described (De Block and Debrouwer, 1992) with slight modifications to allow the staining of whole *Arabidopsis* seedlings. The transgenic plants were incubated at 37 °C in the GUS staining buffer, 50mM $NaHPO_4$, 2mM potassium ferrocyanide, 2mM potassium ferricynide, 0.2% Triton X-100 and 2mM X-Gluc, for 2 h or overnight (14h). A minimum of four independent transgenic lines were used for the histological studies. Untransformed plants were also used as controls.

2.2.4. Development of *AlaAT* over-expressing transgenic lines.

For over-expression of *AlaAT*, the cauliflower mosaic virus 35S (CaMV35S) promoter was used. The construct was designed by introducing a barley AlaAT cDNA (Z26322) into the EcoRI/XbaI sites of plasmid pART7 (Gleave, 1992). The expression cartridge, consisting of the cDNA flanked by the CaMV35S promoter and the octopine synthase gene (*ocs*) 3'-untranslated region, was transferred into the NotI site of the corresponding binary vector, pART27. The construct was then introduced into *Arabidopsis* plants (ecotype C24) using a modified root transformation protocol (Dolferus *et al.*, 1994b). Transformed plants were selected based on kanamycin resistance and homozygous T3 or T4 seeds were used for all experiments.

2.2.5. Isolation of *AlaAT1* knockout line

The *AlaAT1* knockout line was isolated from Salk T-DNA insertion lines (Alonso *et al.* 2003), obtained from the *Arabidopsis* Biological Resource Centre (ABRC), using the PCR screening scheme described on the Salk webpage (http://signal.salk.edu). Primers specific for the left border sequence of T-DNA, LBb1 (5'-GCGTGGACCGCTTGCTGCAACT-3'), and for *AlaAT1* (5'-

GCGTGCTTGGAAGATTCTGGA-3' and 5'-GGAATGGAACTTTTGTCAGGGA-3'), were used for the screening of plants homozygous for the T-DNA insertion. The primers for *AlaAT1* were also used for RT-PCR analysis on the *alaat1-1* line. For the segregation analysis of the kanamycin resistant gene, primers for *NPTII* (5'-CGCTCAGAAGAACTCGTCAAGAA-3' and 5'-TTTGTCAAGACCGACCTGTCC-3') were used for PCR analysis.

2.2.6. Isolation of *AlaAT2* mutant lines

Putative *AlaAT2* mutants were identified by The Canadian TILLING Initiative (CAN-TILL, University of British Columbia, Vancouver, B.C., Canada), and the seeds for the identified mutants were obtained from ABRC (see Appendix 2 for the complete list of the identified putative *AlaAT2* mutants). The obtained putative *AlaAT2* mutants were grown and screened by measuring the AlaAT activity in shoots. Based on the preliminary screening, two putative lines that were homozygous for the point mutation were selected. The point mutation in *alaat2-1* resulted in the amino acid change (Ala → Thr) at 185th position of AlaAT2 protein (A185T), while *alaat2-2* contained mutation resulting in the change (Arg → Lys) at 366th position (R366K). To get rid of other point mutations in these lines, the selected mutants were backcrossed with WT plants for 5 generations. The presence of the point mutation in *AlaAT2* in each backcross generation was checked by CAPS (cleaved amplified polymorphic sequences) marker resulted from the corresponding point mutation. The point mutation in *alaat2-1* (A185T) resulted in the loss of the recognition site for the restriction enzyme, PvuII, while that in *alaat2-2* (R366K) resulted in the loss of the BseRI recognition site. The following primer sets were used to amplify the regions spanning the mutation site; A185T (5'-CACGCAAGTCTTTTGGATGA-3'and 5'-CCGCAAGAACCTAACCAGAA-3'), R366K (5'-GAAAACCAGCGTGACATTGTT-3' and 5'-GCCAGAACCAGGGACTACAA-3'). After five rounds of backcrossing, mutant plants homozygous for the corresponding point mutations were selected. Seeds harvested from the selected homozygous plants were used for the enzyme assays and the metabolite analysis.

2.2.7. Enzyme and metabolite assays

Plants that were hypoxically treated for 24 h or untreated plants were used. AlaAT enzyme extractions and activity measurements were conducted as previously described (Ismond *et al.*, 2003). In brief, harvested tissues were ground in an extraction buffer (5mM EDTA, 1mM DTT, 10mM cysteine, 0.1mM PMSF, 5μM leupeptin and 100mM Tris pH 7.5) with a pinch of sand and PVPP and then centrifuged for 15 min to remove the cell debris. AlaAT activity was measured in the reverse reaction (alanine to pyruvate) in the assay buffer (100mM Tris pH 8.0, 10mM 2-oxoglutarate, 0.28mM NADH, 70mM alanine and 1.2 U/ml lactate dehydrogenase). Alanine was added last to initiate the reaction, and the concomitant decrease of A_{340} by oxidation of NADH was monitored. Protein measurements of all extracts were performed using a Bio-Rad Protein Assay kit (Bio-Rad; http://www.bio-rad.com/) using BSA as standards.

Aerobically (for 0 h treatment) and hypoxically treated plants were used for metabolite extractions. The samples were taken at 2 h, 8 h and 24 h of hypoxic treatment. The stressed plants were subsequently returned to normoxic conditions after 24 h of hypoxic treatment, and samples were taken at 12 h, 24 h and 48 h after the return to normoxia. Metabolite extractions were carried out as described previously (Good and Muench, 1993). Harvested tissues were flash frozen in liquid nitrogen and immediately ground using a mortar and pestle in 0.6M perchloric acid with a pinch of sand. The perchloric acid extracts were then neutralized with 3M KOH and stored at -70ºC until used for the assay. Levels of pyruvate, lactate, ethanol, alanine and glutamate were measured by enzyme-linked assays using a 96 well ELISA plate reader (SpectraMax+) (Molecular Devies, Sunnyvale, Ca, USA; http://www.moleculardevices.com) at room temperature (Ismond *et al.*, 2003). Samples were spiked with known amounts of metabolites to test the accuracy of the assay. Triplicates were used in all experiments, and the experiments were repeated at least 2 times for each time point.

2.2.8. Western blot analysis

Soluble proteins were extracted from shoots and roots using the enzyme extraction buffer (see above) and then separated on a SDS polyacrylamide gel (7.5% running gel/ 4% stacking gel) using a Mini-Protein II Electrophoresis Cell (Bio-Rad).

Proteins were then transferred to a nitrocellulose membrane using a Mini Trans-Blot Cell (Bio-Rad) according to the manufacturer's specifications. The primary antibody raised against purified barley AlaAT2 (Good and Muench 1992, Muench and Good 1994) was used. Antibody staining was performed with an ECL Western detection kit (Amersham Biosciences; http://www5.amershambiosciences.com) using a Western Blocking Reagent from Roche Applied Science (http://www.roche-applied-science.com) instead of non-fat dry milk to improve the specific binding of the primary antibody to the AlaAT proteins.

2.2.9. Root tip survival assay

To assess the anaerobic tolerance of the plants, a root tip survival assay was performed as described previously (Ellis *et al.*, 1999) with slight modifications. Sterilised seeds were sown on nylon mesh strips overlying 0.5x MS media solidified with 1 % agar and supplemented with 1% sucrose. Plates were placed standing upright to allow growth of the roots only on the surface of the medium. After 7 days, the nylon mesh strips supporting the seedlings were transferred to liquid 0.5x MS media containing 1% sucrose and timentin (25mg/l) that had been previously gassed with full nitrogen gas or 0.1% oxygen gas (balanced with nitrogen) for 15 min. Half-cut microfuge tubes were placed inside the pétri dishes to slightly lift the lids and to ensure rapid gas exchange. The plates were then stacked into anaerobic jars and flushed with full nitrogen gas or 0.1% oxygen gas for an additional 15 min. The anaerobic jars were kept in the dark for the duration of hypoxic treatment. At the end of the stress treatment, 15 plants per plate were randomly selected and transferred to square pétri dishes containing 0.5x MS media with 1% agar and allowed to recover for 1 week. Root tip survival, based on the ability of the primary root tip to re-grow, was scored after 2 days from transferring seedlings to the new square plates. At the end of the recovery period, shoot and root survival, as well as shoot and root weights, were recorded. Triplicate samples were used in all experiments and the experiments were repeated 3 times.

2.2.10. Alanine supplementation test

To assess the plants' ability to utilise alanine as nitrogen source, plants were grown on controlled 0.5x MS media (0.6% agar) with varying concentrations of inorganic nitrogen (0-10mM inorganic nitrogen, KNO_3: NH_4NO_3 = 1:1); with or without 5mM alanine supplementation. Two sucrose concentrations, 3% and 0.3%, were also used. Typically, 22 seeds of the Ox*AlaAT* lines or the *alaat1-1* line were co-plated with corresponding WT seeds on the same plate. After 2 weeks of growth, the plates were photographed and the fresh weight and dry weight of the above ground tissues of 20 plants were measured. Triplicates were used in all experiments and the experiments were repeated 3 times.

2.3 Results

2.3.1. *Arabidopsis AlaAT* gene family and their expression profiles

The *Arabidopsis AlaAT* gene family consists of four *AlaAT* genes (Liepman and Olsen 2003; Igarashi *et al.*, 2003). These four genes are subdivided into two groups, genes encoding AlaAT (*AlaAT1*; At1g17290 *and AlaAT2;* At1g72330) and those encoding GGAT (*GGAT1;* At1g23310 *and GGAT2;* At1g70580). *Arabidopsis AlaAT1* and *AlaAT2* have high sequence identity to the previously characterized *AlaAT* genes of other plant species such as barley (Muench and Good, 1994), maize (Muench *et al.*, 1998) and rice (Kikuchi et al., 1999). Igarashi *et al.* (2003) have analyzed the expression profile of each member of the *Arabidopsis AlaAT* gene family using real-time RT-PCR and have shown expression of *AlaAT1* in both leaves and roots as well as the strong expression of *GGAT1* in leaves. To confirm similar gene expression profiles in our growth system, we examined expression levels of each gene using total RNA isolated from 17-day-old seedlings. Strong expression of *AlaAT1* was observed in both roots and shoots, whereas weak expression of *AlaAT2* was detected only in shoots (Figure 2.1). The expression of *GGAT1* was observed in shoot tissue but not in root tissue (Figure 2.1). These results indicate that *AlaAT1* is the major gene expressed in roots among the four members of the *Arabidopsis AlaAT* gene family.

2.3.2. Expression of *AlaAT* genes are induced by low oxygen stress

The hypoxic induction of *Arabidopsis AlaAT1* was previously shown in microarray studies (Klok *et al.*, 2002; Liu *et al.*, 2005; Loreti *et al.*, 2005). These experiments demonstrated that low oxygen stress rapidly induced *AlaAT1* expression upon the onset of the stress (Figure 2.2a). A dramatic induction of the well-characterized anaerobically induced gene, *ADH1*, was also observed in the microarray experiment (Figure 2.2a). We analyzed the expression profiles of the *AlaAT* gene family in response to low oxygen stress. To do this, we performed semi-quantitative RT-PCR on total RNA samples isolated from plants subjected to various lengths of hypoxic stress (Figure 2.2b). In roots, induction of both *AlaAT1* and *AlaAT2* was observed at 2 h of hypoxia and peaked at 8 h (>4-fold increase). In shoots, this up-regulation occurred more rapidly and was obvious for both genes at 2 h of hypoxic stress treatment. These results demonstrate that both *AlaAT* genes are induced during hypoxia. For *GGAT1,* there was no detectable message in root tissues, however, an induction was observed in shoot tissues at 8 h of hypoxia. The induction of GGAT1 in shoot tissues contrasts with what was observed in the microarray experiments (Figure 2.2a). Since the different experiments were conducted at similar time points (6h versus 8h), the discrepancy between these results is unclear. For GGAT2, expression was nearly undetectable at all time points in roots and shoots.

2.3.3. *AlaATs* are preferentially expressed in vascular tissues

Transgenic plants expressing the *β-glucuronidase* (*GUS*) reporter gene under the control of *AlaAT1* and *AlaAT2* promoters were stained for GUS activity under both aerobic and hypoxic conditions.

Under aerobic conditions, *pAlaAT1:GUS* transgenic plants showed strong GUS expression in the vascular tissues of both roots and shoots (Figure 2.3a, 2.3e). GUS staining in roots was noticeably fainter in the sections closer to the tip of the roots and was absent in root tips as well as in the lateral root primordia (Figure 2.3a, 2.3b). In leaves, preferential staining of vascular tissues was observed, but staining was also seen throughout the young rosette leaves (Figure 2.3e). *pAlaAT2:GUS* transgenic plants showed similar expression patterns in shoots, but the coloration was fainter (Figure 2.3k). In addition, cotyledons and the first pair of rosette leaves were not stained intensely (Figure 2.3k). In roots, the vascular *GUS* expression was limited to the upper portions of the primary root (data not shown). Typically, *GUS* expression was absent in most of the root system including the lateral roots, the lateral root primordia and the root tips (Figure 2.3g, 2.3h). Non-transgenic plants showed no background GUS activity (data not shown).

In hypoxically treated plants, similar GUS staining patterns to those of untreated plants were observed for both *pAlaAT1:GUS* and *pAlaAT2:GUS* transgenic plants (Figure 2.3c, 2.3i). To detect low-level GUS activity, GUS staining was also performed overnight (14h duration). When stained for a longer time period, more intensified vascular staining of the roots became apparent for *pAlaAT1:GUS* plants (Figure 2.3d), but it was still faint for *pAlaAT2:GUS* plants (Figure 2.3j). The root tips remained unstained for both types of transgenic plants. In shoots, GUS expression occurred predominantly in the vascular tissues, but weak staining was also detected in non-vascular leaf tissues for both *pAlaAT:GUS* and *pAlaAT2:GUS*. For better resolution, the leaves of 20-day-old plants stained overnight are shown (Figure 2.3f, 2.3l).

To test whether the promoter fragments used in this study were capable of expressing *GUS* similar to the native promoters, we confirmed the similar hypoxic induction patterns of *GUS* to those of *AlaAT1* or *AlaAT2* (Figure 2.4). The results indicate that the expression patterns of *GUS* seen in Figure 2.3 are reflective of the tissue specificity of the two endogenous *AlaAT* genes.

2.3.4. Isolation of an *alaat1-1* mutant and transgenic plants over-expressing a barley *AlaAT*

We screened Salk T-DNA insertion lines (Alonso et al., 2003) in search of knockout mutants for *AlaAT1* and *AlaAT2* (see Table 2.1 for the complete list of the Salk lines screened in this study). We identified one mutant line containing a T-DNA insertion in *AlaAT1* between the 8th intron and 9th exon (Figure 2.5a). Left border sequences were found on both sides of the insertion junction, suggesting the insertion of inverted repeats of the T-DNA. RT-PCR analysis indicated the absence of normal transcript in this line (Figure 2.5b), thus we designated the mutant line *alaat1-1*. An aberrant product was amplified when using total RNA isolated from the shoots of *alaat1-1* and appeared to be derived from non-specific annealing of the primer to a T-DNA region, based on sequencing results (data not shown). Western blot analysis confirmed the absence of AlaAT protein in the *alaat1-1* line (Figure 2.5c). To confirm the number of loci containing T-DNA insertions in *alaat1-1,* the knockout mutant was crossed with non-transgenic plants. Segregation of the kanamycin resistance gene, *NPTII*, was followed by PCR in the F2 population since *NPTII* was silenced in the mutant line. *NPTII* segregated in a 3:1 ratio and was detected only in plants containing the T-DNA insertion within *AlaAT1* (data not shown), suggesting that the *alaat1-1* line contained the T-DNA insertion only in one locus.

For *AlaAT2*, the only T-DNA insertions found were in the promoter region of the gene (Table 2.1). These insertions did not affect the expression of *AlaAT2* under either aerobic or hypoxic conditions (data not shown).

To produce transgenic plants with higher levels of AlaAT activity, we over-expressed a barley *AlaAT* cDNA under the control of CaMV 35S promoter. Twenty independent transgenic lines were carried through to the T3 and T4 generations. Segregation of *NPTII* indicated that, of the lines selected (Ox*AlaAT#10*, *#15*, *#18*, *#20,* prefix "Ox" for over-expression) based on the preliminary enzyme activity assay, all except *OxAlaAT#18* contained a single insertion of the transgene. The Ox*AlaAT#18* line appeared to have the transgene inserted in two loci (data not shown). We confirmed the presence of the barley *AlaAT* transcript by RT-PCR (Figure 2.5d) and that of the protein by Western blot analysis (Figure 2.5e). These results indicated that the transgene was actively transcribed and translated in the selected Ox*AlaAT* lines. However, the expression in *OxAlaAT#15* was not uniform between different tissue types despite using a constitutive promoter. This may indicate a tissue specific position effect.

2.3.5. Manipulated AlaAT enzyme activity in *alaat1-1* and *OxAlaAT* lines

We measured AlaAT enzyme activity in *alaat1-1* and the Ox*AlaAT* lines under both aerobic and hypoxic conditions (Figure 2.6). Under aerobic conditions, the enzyme activity in the roots of *alaat1-1* plants was negligible, whereas the activity in the shoots was reduced to 20-30% of WT activity. The hypoxic induction of AlaAT activity was only seen in roots of WT plants after 24 h of hypoxia (approximately 1.6-fold). The results indicated that the T-DNA insertion in the *AlaAT1* resulted in a dramatic decrease in overall AlaAT activity. Furthermore, the absence of activity in roots of *alaat1-1* suggests that AlaAT1 is the isoenzyme responsible for nearly all AlaAT activity in roots of *Arabidopsis*. The low-level AlaAT activity detectable in shoots of *alaat1-1* could be mostly attributable to GGAT1, which was shown to be responsible for the portion of the AlaAT activity delectable in photosynthesizing tissues (approximately 40%) (Igarashi et al., 2003).

The three selected Ox*AlaAT* lines showed significant increases in AlaAT activity (Figure 2.6). Under aerobic conditions, approximately 3.5 to 7-fold increases in activity were seen in roots of the Ox*AlaAT* plants. AlaAT activity levels were similarly elevated in shoots of the Ox*AlaAT* lines (approximately 4.5 to 10- fold increases). When subjected to hypoxic stress, AlaAT activity in roots increased approximately 1.5-fold in WT plants, whereas smaller inductions were observed in roots of the Ox*AlaAT* lines (less than 1.2-fold increase). In the shoots, the hypoxic induction of AlaAT activity was not evident in WT plants and activity decreased in the *OxAlaAT* lines under hypoxia, perhaps as a result of a hypoxically triggered reduction in CaMV35S promoter activity or the reduction in translation of barley *AlaAT* mRNA.

2.3.6. Metabolic and physiological analysis of *alaat1-1* and *OxAlaAT* lines

To investigate how the mutation in *AlaAT1* and over-expression of barley *AlaAT* affect metabolic profiles, we analyzed the levels of several metabolites in *alaat1-1* and the Ox*AlaAT* lines (Figure 2.7). For these assays, plants were grown for 17 days and then subjected to hypoxia for various lengths of time. After 24 h of hypoxic treatment, plants were returned to aerobic conditions for up to 48 h. Plant samples taken at various time points during the hypoxic treatment and the subsequent recovery period were used for metabolite assays.

For ethanol and lactate, *alaat1-1* and the WT plants showed almost identical patterns of accumulation in the external media during hypoxic stress (Figure 2.7a). Upon return to aerobic conditions, both metabolites disappeared from the media at the same rate in both *alaat1-1* and WT. A rapid increase in alanine concentration under hypoxia was observed in both *alaat1-1* and WT plants (Figure 2.7a). Typically, alanine increased at a faster rate in roots than in shoots. In roots of *alaat1-1*, alanine increased more rapidly than in WT plants, and significantly higher levels of alanine, relative to WT levels, was found at 2 h of hypoxia (1.34-fold). *alaat1-1* plants also contained higher levels of alanine at 24 h of hypoxia, but this difference was not statistically significant and could not be repeated in other independent experiments. Upon return to aerobic conditions, alanine concentrations gradually decreased. During the recovery period, the decrease in alanine was partially inhibited in *alaat1-1*, and significantly higher concentrations of alanine (1.59-fold) remained in roots of *alaat1-1* at 24 h after return to normoxia. In shoots, significantly higher levels of alanine (1.74-fold) were detected in *alaat1-1* than WT plants at 24 h after return to normoxia. The partial inhibition in the decline of alanine concentrations in *alaat1-1* plants was verified in separate experiments with additional time points over the 48 h recovery period (Figure 2.7b). The results showed that the decrease in alanine, upon return to normoxia, did not start in *alaat1-1* until after the first 6 h of recovery, indicating the delay in alanine catabolism in the initial phase of the recovery from hypoxia. In this experiment, the *alaat1-1* plants contained more alanine than WT at all times during the recovery period in both shoots and roots (Figure 2.7b).

OxAlaAT lines produced and accumulated ethanol and lactate in the external media similar to WT plants during hypoxia (Figure 2.8). The accumulated metabolites disappeared from the media during the post-hypoxic period. Alanine concentrations increased at a similar rate in the Ox*AlaAT* lines and WT plants during hypoxic treatment (Figure 2.8). During the recovery, one transgenic line, *OxAlaAT#10,* displayed a more rapid decrease of alanine in the roots, however; this was not observed in the other Ox*AlaAT* lines. The level of glutamate was generally unaffected throughout the time course experiment in both Ox*AlaAT* lines and WT plants, although the Ox*AlaAT* lines contained more glutamate during the recovery period.

To evaluate low oxygen stress tolerance in *alaat1-1* and the Ox*AlaAT* lines, we subjected the plants to either anoxic (100% N_2) or hypoxic conditions (0.1% oxygen, 99.9% N_2) for various lengths of time and then scored the survival of the primary root tip based on its ability to maintain growth when returned to normoxic conditions. The decline in viability of the root tips over the period of anoxic stress was similar in *alaat1-1* and WT plants (Figure 2.9). The root tips of Ox*AlaAT* lines also behaved similar to the WT plants (Figure 2.9). Hypoxic stress treatment showed milder effects on the root tip survival, but did not result in differences between *alaat1-1* and WT plants or between *OxAlaAT* lines and WT plants. The shoot and root weight of plants that recovered from anoxic or hypoxic conditions were not significantly different between *alaat1-1* and WT plants or between Ox*AlaAT* lines and WT plants (data not shown).

2.3.7. The ability to utilize exogenously supplied alanine is affected in *alaat1-1*.

Since the metabolite assays suggested that AlaAT generally converts alanine to pyruvate *in vivo*, we hypothesized that the *alaat1-1* knockout would not be able to utilize alanine as a nitrogen source as effectively as WT plants. To test this hypothesis, plants were grown on MS media containing various concentrations of inorganic nitrogen (KNO_3: NH_4NO_3 = 1: 1) with or without supplementation of alanine. When grown without alanine supplementation, no differences were observed between *alaat1-1* and WT plants, however; when grown on low N media with alanine supplementation, *alaat1-1* plants showed reduced growth compared to WT plants (Figure 2.10, Table 2.2). On alanine-supplemented media, WT plants grew approximately 1.3 to 1.5-fold larger than *alaat1-1* plants. It should be noted however that the *alaat1-1* line was still capable of utilizing the supplied alanine as indicated by the improved growth of *alaat1-1* plants on low-N media, when supplemented with alanine. The Ox*AlaAT* lines did not show any differences relative to WT plants when grown on these media. The results from a representative line (*OxAlaAT#20*) are shown in Table 2.2.

2.3.8. Characterization of *alaat2* mutants.

From the putative *alaat2* mutants identified by TILLING, two lines were selected and backcrossed with WT for 5 generations. The homozygous plants obtained after 5 rounds of backcross were used for the preliminary characterization of *AlaAT2*. In addition, *alaat2* mutants were crossed with *alaat1-1* to obtain a double mutant deficient in both *AlaAT1* and *AlaAT2*.

Using these lines, AlaAT enzyme activity was measured in roots and shoots of 17-day-old seedlings. In both of the *alaat2* mutants, a small reduction in enzyme activity was only observed in shoots but not in roots (Figure 2.11a). The AlaAT activity of *alaat1-1* and the double mutant, *alaat1-1/alaat2-1,* was similar (Figure 2.11a). The results obtained from these lines were thus consistent with the stronger expression of *AlaAT2* in shoots than in roots demonstrated by semi-quantitative RT-PCR (Figure 2.1) and by promoter:*GUS* study (Figure 2.3). The small decline in the AlaAT activity in the putative *alaat2* mutants suggests that the point mutation in each line affected the activity of the AlaAT2 protein. Taken together, it appears that AlaAT2 is responsible for only a small AlaAT activity in shoots at least under normal conditions.

The alanine accumulation during hypoxia and post-hypoxia was also monitored in these lines. In both *alaat2* mutants and the double mutant, the levels of alanine increased in both shoots and roots during hypoxic stress (Figure 2.11b). The double mutant also showed similar alanine accumulation during hypoxic treatment (Figure 2.11b). Upon return to normoxic conditions, the breakdown of alanine was partially inhibited in both of the *alaat2* mutants and in the double mutant, evidenced by the higher levels of alanine remained in roots at 12 h after return to normoxia (Figure 2.11b). However, the inhibition in the alanine catabolism in the mutant lines were less clear in shoots compared to that in roots (Figure 2.11b). These results suggest that AlaAT2 play a redundant role as AlaAT1 during the recovery from low oxygen stress.

2.4. Discussion

The hypoxic induction of *AlaAT* is a well-known response of plants subjected to low oxygen stress. The different physiological roles of AlaAT are suggested by the diverse induction profiles of the genes by several different stresses and by the presence of multiple isoforms in distinct subcellular compartments, including the cytosol, mitochondria and peroxisome (Biekmann and Feierabend, 1982; Liepman and Olsen, 2004). In *Arabidopsis*, four AlaAT isoenzymes, each encoded by a member of the *AlaAT* gene family have been described previously (Liepman and Olsen, 2003, Igarashi *et al.*, 2003). In this study, we have looked at expression of *AlaAT1* and *AlaAT2* and attempted to elucidate the role of AlaAT through a functional analysis of T-DNA *AlaAT1* knockout mutant, putative *AlaAT2* mutants and transgenic lines over-expressing barley *AlaAT* (Ox*AlaATs*) in *Arabidopsis.*

The transcriptional induction of *AlaAT* genes in response to low oxygen conditions has been demonstrated in barley (Muench and Good, 1994), maize (Muench *et al.*, 1998), and *Medicago truncatula* (Ricoult *et al.*, 2005). In *Arabidopsis,* several microarray experiments have shown the up-regulation of *AlaAT1*, in response to hypoxic stress, however; the response of *AlaAT2* to hypoxia remained uncertain (Klok *et al*., 2002; Liu *et al*., 2005; Loreti *et al*., 2005). In one experiment, small up-regulation of *AlaAT2* was detected by microarray analysis, but this could not be repeated with real-time RT-PCR (Liu *et al*., 2005). In another study, the probes corresponding to *AlaAT2* were not present on the Affymetrix ATH1 chip used in the experiment (Loreti *et al*., 2005). In the current study, we showed hypoxic induction of both *AlaAT1* and *AlaAT2* (Figure 2.2b). As to the induction profiles, our results demonstrate that transcript levels of both *AlaAT1* and *AlaAT2* increase quickly, plateau for a short period of time, and then slowly decrease. These induction profiles were similar to that of *ADH1* in *Arabidopsis* (Dolferus *et al*., 1994a, Kürsteiner *et al*., 2003). However, as with *ADH1*, the degree of transcriptional induction of the *AlaAT* genes was significantly higher than the concomitant increase in enzyme activity (Figure 2.2b, Figure 2.6). In general, the increase in enzyme activity required relatively longer stress treatments (>24-48 h). The slight increase of AlaAT activity after 24 h of hypoxia (Figure 2.6) is consistent with the increases in enzyme activity observed at 24 h of hypoxic treatment in barley (Good and Crosby, 1989), maize (Muench et al., 1998) and soybean (Sousa and Sodek, 2003). Moreover, the increase in AlaAT activity was limited to roots, perhaps suggesting the inefficient translation of both *AlaAT* transcripts in shoots under low oxygen conditions.

The tissue specificity of both *AlaAT* transcripts was analyzed by developing *Arabidopsis* transgenic plants expressing *GUS* under the control of either the *AlaAT1* or *AlaAT2* promoter. The analysis of the transgenic plants demonstrated that *AlaAT1* is expressed at higher levels in the vascular tissues of both shoots and roots, while *AlaAT2* is expressed predominantly in shoot vascular tissues. Similarly, *AlaAT* expression within vascular tissues has been shown in rice by GUS transcriptional fusions and by *in situ* hybridization (Kikuchi *et al*., 1999). In this study, the absence of expression of both *Arabidopsis AlaAT* transcripts within root tips is noteworthy when compared to the expression of rice *AlaAT* in the root tip (Kikuchi *et al*., 1999) and that of other genes encoding ANPs, such as *Arabidopsis ADH1* (Dolferus *et al*., 1994b). Furthermore, the high expression of *AlaAT1* also occurs in pollen as shown in the AtGenExpress expression atlas (Schmid *et al*., 2005). Pollen expression is also known for other ANPs, including pyruvate decarboxylase (PDC) and ADH, indicating the operation of an anaerobic-like metabolism in germinating pollen (Gass *et al.*, 2005). Hypoxic stress did not affect the GUS staining patterns, although apparent transcriptional inductions of both *AlaAT* and *GUS* driven by either of the *AlaAT* promoter were confirmed by semi-quantitative RT-PCR (Figure 2.4). This inconsistency may be attributed to the inefficient translation of GUS mRNA, which could be due to absence of cis-elements from the GUS transcript that are required for translation under low oxygen stress. Alternatively, this could result from the fact that the GUS staining occurs in the same tissues under hypoxic and normoxic conditions and any increase in staining intensity would be minor, as is the case for the increased AlaAT enzyme activity.

The changes in metabolite levels during the period of hypoxia or anoxia have been closely monitored in a number of studies in the past. The pronounced increase in alanine has been well documented in plants subjected to low oxygen stress (Streeter and Thompson, 1972; Good and Muench, 1993; Fan et al., 1997, Sousa and Sodek, 2003). However, the metabolic changes upon return to aerobic conditions have not received the same attention. In this study, the products of anaerobic metabolism (ethanol, lactate and alanine) were measured during the post-hypoxic period. A steady decrease in alanine concentration was observed during the post-hypoxic period. Sousa and Sodek (2003) have also shown a similar but more rapid fall of alanine within 24 h post-hypoxia.

The involvement of AlaAT in hypoxic alanine production has been long speculated, however compelling evidence has been missing. We addressed this question by analysing the responses of *alaat1-1*, *alaat2* mutants and the Ox*AlaAT* lines to hypoxia. Under this stress condition, *alaat1-1* plants accumulated high levels of alanine, and the increase in alanine content was even more rapid in *alaat1-1* plants than in WT plants (Figure 2.7a). On the other hand, *alaat2* mutant and Ox*AlaAT* lines accumulated alanine as rapidly as WT plants in response to low oxygen stress (Figure 2.7b). These results refute the hypothesis that the main role of AlaAT under hypoxia is the production of alanine. Instead, the partial inhibition of alanine breakdown in *alaat1-1* and *alaat2* mutant plants upon return to normoxic conditions suggests that AlaAT plays a role in the conversion of alanine during the post-hypoxic period (Figure 2.7a). Similarly, Sousa and Sodek (2003) concluded that the hypoxic induction of AlaAT is important to reduce elevated alanine levels to normal in a relatively short period of time upon return to normoxia. Particularly, the retainment of higher levels of alanine during the recovery period observed in *alaat2* mutants was a surprising result since the decrease in AlaAT activity in the *alaat2* mutants was very small (Figure 2.11). It can be hypothesized that the transcriptional up-regulation of *AlaAT2* result in the small increase in AlaAT2 protein in mitochondria, thus exerting a stronger effect on the alanine catabolism compared to the putatively cytosolic AlaAT1. When subjected to hypoxic stress, plants are known to express genes whose products have functions during the subsequent recovery, "anticipating" the return to normoxic conditions (Drew, 1997). The genes encoding enzymes responsible for the detoxification of reactive oxygen species generated during re-exposure to ambient oxygen (superoxide dismutase, peroxidase, ascorbate peroxidase, monodehydroascorbate reductase and glutathione reductase) are good examples (Klok *et al.*, 2002, Geigenberger, 2003).

Manipulation of AlaAT activity did not alter the survival of the primary root tip under low oxygen conditions. The root tips of *alaat1-1* plants and the over-expressing lines showed similar susceptibility to anoxic stress as the corresponding WT plants (Figure 2.10). Hypoxic stress caused milder effects on these plants; however, there was no significant difference in their tolerance to hypoxic stress. These results suggest that AlaAT activity may not be necessary for root tip survival under low oxygen stress. The absence of GUS staining in the root tips (Figure 2.3) also support a similar conclusion.

Since *alaat1-1*, *alaat2* mutants and Ox*AlaAT* lines are perfectly viable and are phenotypically normal, the physiological role of AlaAT still remains ambiguous. However, the reduced growth of *alaat1-1* plants on media supplemented with alanine suggests that AlaAT functions to convert excess alanine to pyruvate. This was also supported by the delayed decline of alanine in *alaat1-1* and *alaat2* plants during the post-hypoxic period. A similar conditional phenotype was observed in a mutant for cytosolic aspartate aminotransferase (*aat2-2*) where addition of aspartate (20mM) to the media impaired root development (Miesak and Coruzzi, 2002). In their experiment, the inability of the *aat2-2* mutant to negate the toxic effect of excess aspartate was most likely the cause of the reduced growth of *aat2-2* plants (Miesak and Coruzzi, 2002). In this study, we tested the impact of alanine supplementation on low-nitrogen media, thereby forcing the plants to utilize the amino N of alanine for growth. The inefficient utilization of supplied alanine in the *alaat1-1* line is the likely cause of the reduced growth of the *alaat1-1* plants. The results also indicate that alanine was less efficient as a source of N, when compared to inorganic N. Indeed, 5mM alanine resulted in growth which was equivalent to approximately 1.8-2.0mM of inorganic N (Table 2.2). It has been demonstrated that amino acids can be an important form of organic nitrogen utilized by plants (Lipson and Näsholm, 2001, Thornton and Robinson, 2005). Our experiment demonstrated that *Arabidopsis* possesses the ability to uptake and utilize externally supplied alanine, however; the ability of plant roots to utilize amino acids in agricultural conditions remains uncertain.

Although the activity of AlaAT2 appears to be minor, the predicted mitochondrial localization (Liepman and Olsen, 2003) suggests this isoenzyme is important in the targeted organelle. Under low oxygen stress, the membrane structure of mitochondria is affected and mitochondria play a key role in the perception and signaling of falling oxygen levels within the cell (Vartapetian *et al.*, 2003, Subbaih and Sachs, 2003).

In summary, our results indicate that AlaAT1 catalyzes primarily the conversion of alanine to pyruvate. The normal alanine levels found in *alaat1-1*, *alaat2* mutants and *alaat1/alaat2* double mutant under aerobic conditions also suggests that *Arabidopsis* has multiple pathways for production and breakdown of alanine, and normal levels of alanine can be maintained without functional *AlaAT1* and *AlaAT2*. Over-expression of *AlaAT* also did not alter alanine levels in the transgenic plants. This suggests that the AlaAT reaction is not the rate-limiting step for either alanine synthesis or breakdown. Considering the vascular expression of both *AlaAT* transcripts, AlaAT activity may be important for the translocation of either alanine or pyruvate and may facilitate the maintenance of carbon-nitrogen balance throughout plants.

Table 2.1. Summary of the screening of Salk T-DNA lines.

The T-DNA insertions in each line were confirmed by PCR analysis. The actual insertion sites were confirmed by sequencing the gel purified PCR products. AlaAT activity was measured in shoots and roots of each line. 17-day-old seedlings grown as described in Experimental procedures were used for the enzyme activity assay. (n/a: not available, n.d.: not detected,)

Genes	Locus No.	T-DNA insertion lines	Insertion sites	Enzyme activity (relative to WT)	
				Shoots	Roots
AlaAT1	*At1g17290*	Salk_107660	No	n/a	n/a.
		Salk_107662	9th Exon	21.0 %	n.d.
		Salk_107664	No	n/a	n/a
AlaAT2	*At1g72330*	Salk_105899	Promoter	91.0 %	94.5 %
		Salk_004925	Promoter	99.0 %	94.0 %
		Salk_083084	Promoter	81.5 %	95.0 %
GGAT1	*At1g23310*	Salk_064982	3' UTR	85.0 %	91.0 %
GGAT2	*At1g70580*	Salk_042954	4th Intron	94.0 %	88.5 %
		Salk_011617	12th Exon	93.0 %	93.0 %

Table 2.2. Fresh weights of plants grown on alanine supplementation media.

Fresh weights of 2-week-old plant grown on nitrogen-controlled 0.5x MS media with or without supplementation of 5mM alanine were measured. Values shown are the means of triplicate plates with ± S.E. ** indicates P<0.01 (Student's t-test). The experiments were repeated with similar results.

		0mM N	0.4mM N	2mM N	10mM N
Col (WT)	0mM Ala	4.0 ± 0.2	25.3 ± 0.6	87.4 ± 5.9	237.7 ± 9.8
alaat1-1		4.0 ± 0.8	25.0 ± 0.7	82.5 ± 2.0	232.3 ± 11.9
Col (WT)	5mM Ala	84.6 ± 0.8	133.1 ± 2.1	177.0 ± 2.7	245.9 ± 2.8
alaat1-1		** 57.6 ± 2.0	** 99.4 ± 4.3	**134.2 ± 3.7	225.3 ± 11.6
C24 (WT)	0mM Ala	3.3 ± 0.3	26.6 ± 0.2	95.1 ± 6.5	175.0 ± 15.4
Ox*AlaAT#20*		3.9 ± 0.1	26.2 ± 1.2	87.6 ± 4.7	198.2 ± 11.8
C24 (WT)	5mM Ala	81.3 ± 1.9	97.9 ± 4.8	129.8 ± 3.3	215.6 ± 13.1
Ox*AlaAT#20*		74.9 ± 2.3	101.2 ± 4.5	141.1 ± 6.3	212.4 ± 2.6

(mg/20 plants)

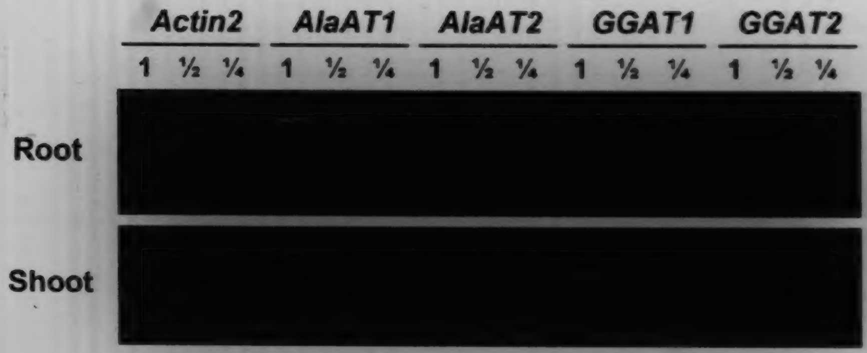

Actin2
AlaAT1
AlaAT2
GGAT1
GGAT2
1 ½ ¼ 1 ½ ¼ 1 ½ ¼ 1 ½ ¼ 1 ½ ¼
Root
Shoot

Figure 2.1. Semi-quantitative RT-PCR of *AlaAT* gene family.

Total RNA isolated from shoots and roots of 17-day-old plants were used. PCR was carried out with 2- or 4-fold diluted aliquots of the synthesized first strand cDNA (lane ½ and ¼) as well as undiluted aliquots (lane 1).

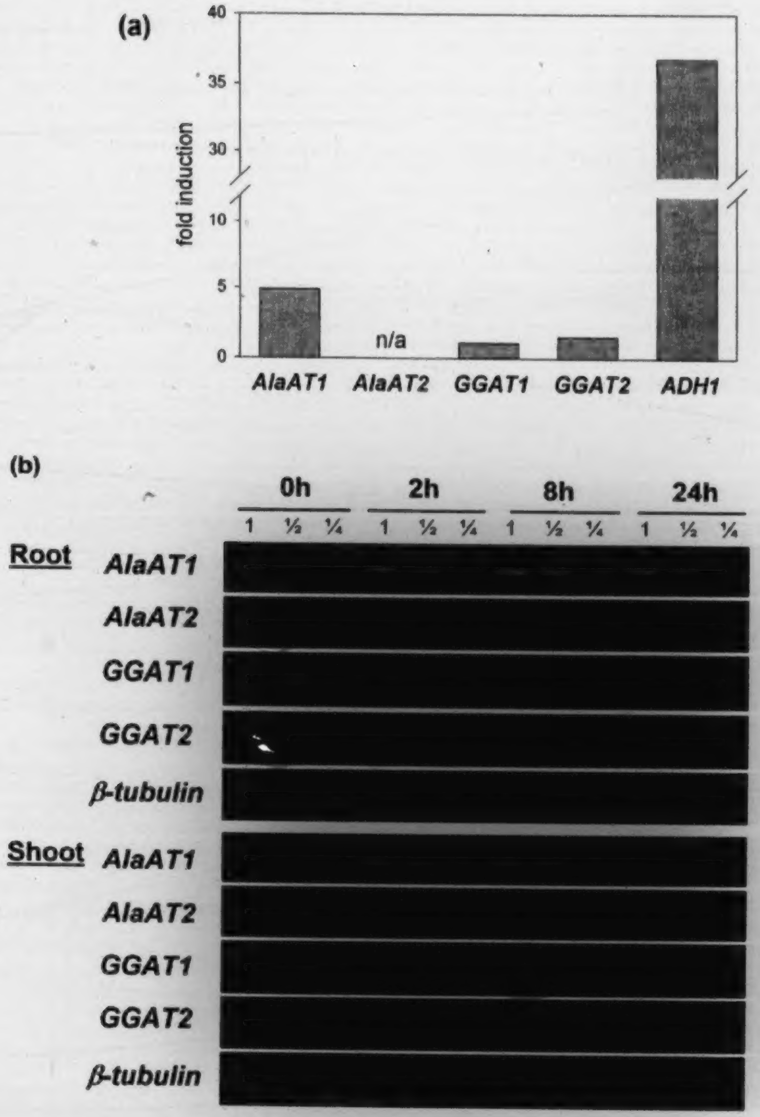


Figure 2.2. Hypoxic induction profiles of *AlaATs*.

(a) Microarray experiments showing the induction of *AlaAT1* and *ADH1* in response to anoxia. The data were taken from the microarray experiment of Loreti et al. (2005). RNA samples for the microarray were extracted from anoxically treated (6 h) and untreated 4-day-old *Arabidopsis* seedlings. The expression levels of each gene were compared between the two treatments. (n/a: not available). (b) Semi-quantitative RT-PCR showing the increased expressions of *AlaATs* transcripts in roots and shoots in response to hypoxic stress. Total RNA was extracted from plants that have been hypoxically treated for the indicated period. PCR was carried out with 2- or 4-fold diluted aliquots of the synthesized first strand cDNA (lane ½ and ¼) as well as undiluted aliquots (lane 1).

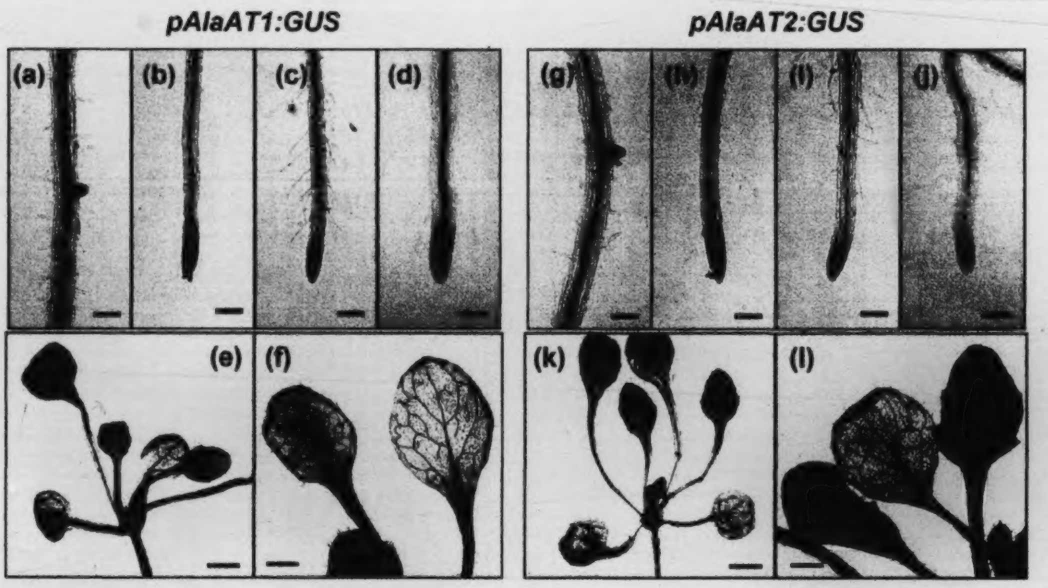


Figure 2.3. Tissue specific expression of *AlaATs*.

Transgenic plants expressing *GUS* under the control of *AlaAT1* and *AlaAT2* promoters were stained for GUS activity. Untreated 14-day-old plants stained for 2 h are shown unless otherwise indicated. (a, b) root (c) root of hypoxically treated plant. (d) root of 20-day-old plant stained for 14 h. (e) shoot. (f) leaves of 20-day-old plant stained for 14 h. (g, h) root. (i) root of hypoxically treated plant. (j) root of 20-day-old plant stained for 14 h. (k) shoot. (l) leaves of 20-day-old plant stained for 14h. Bars in the root pictures and shoot pictures are 0.1mm and 1.0mm, respectively.

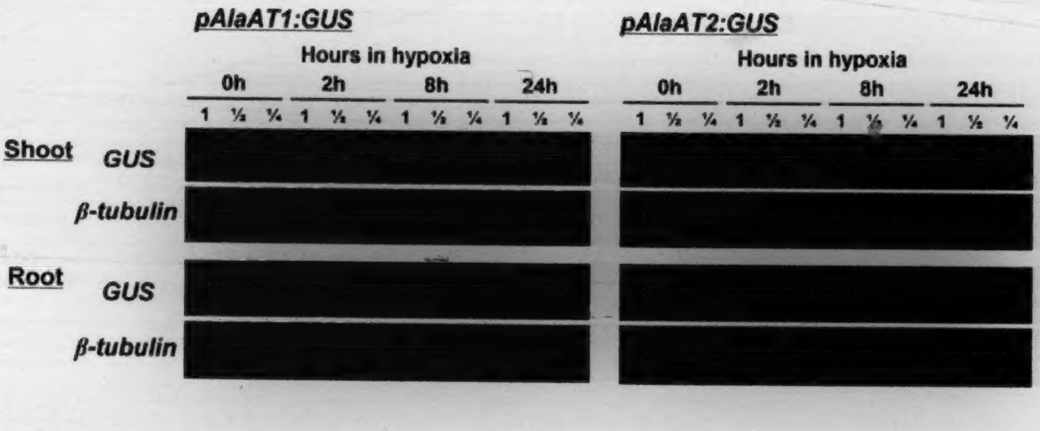

pAlaAT1:GUS
pAlaAT2:GUS
Hours in hypoxia
Hours in hypoxia
0h
2h
8h
24h
0h
2h
8h
24h
1 ½ ¼ 1 ½ ¼ 1 ½ ¼ 1 ½ ¼
1 ½ ¼ 1 ½ ¼ 1 ½ ¼ 1 ½ ¼
Shoot
GUS
β-tubulin
Root
GUS
β-tubulin

Figure 2.4. Semi-quantitative RT-PCR showing the hypoxic induction of *GUS* driven by *AlaATs* promoters.

Total RNA was extracted from the transgenic plants that have been hypoxically treated for the indicated period. PCR was carried out with 2- or 4-fold diluted aliquots of the synthesized first strand cDNA (lane ½ and ¼) as well as undiluted aliquots (lane 1).

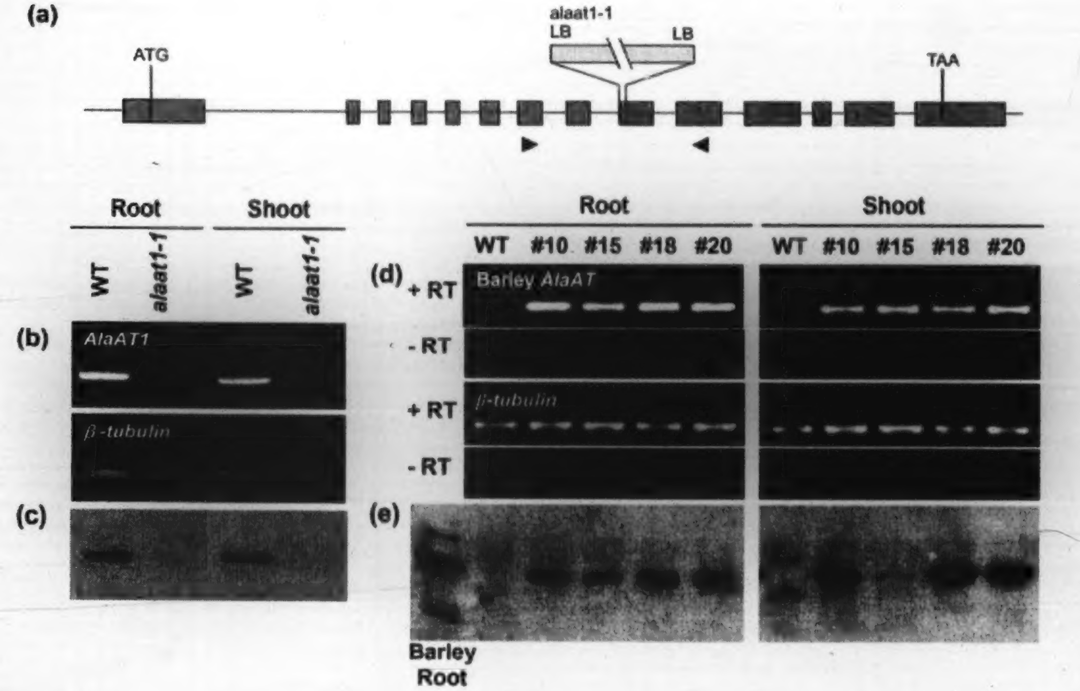

(a)
alaat1-1
LB
LB
ATG
TAA
Root
Shoot
WT
alaat1-1
WT
alaat1-1
(b)
AlaAT1
β-tubulin
(c)
Root
Shoot
WT
#10
#15
#18
#20
WT
#10
#15
#18
#20
(d)
+ RT
Barley AlaAT
- RT
+ RT
β-tubulin
- RT
(e)
Barley
Root

Figure 2.5. Characterization of *alaat1-1* mutant and *AlaAT* over-expressing lines.

(a) The gene structure of *AlaAT1* and the site of T-DNA insertion in the knockout mutant (*alaat1-1*). Exon regions of *AlaAT1* are shown by shaded boxes. (b) RT-PCR analysis showing the absence of normal *AlaAT1* transcript in *alaat1-1*. Total RNA was isolated from roots and shoots of *alaat1-1* and WT plants. PCR was carried out with synthesized first strand cDNA using primers indicated as black triangles in the diagram (a). (c) Western blot analysis on *alaat1-1* and WT. Proteins extracted from shoots and roots of *alaat1-1* and WT plants were used. 10μg proteins were loaded into each lane. (d) RT-PCR analysis showing the expression of barley *AlaAT* in Ox*AlaAT* lines. Total RNA was isolated from roots and shoots of Ox*AlaAT* and WT plants. PCR was carried out with synthesized first strand cDNA (+RT) or DNaseI treated RNA samples (-RT) using gene specific primers. (e) Western blot analysis on Ox*AlaAT* lines and WT. Proteins extracted from shoots and roots were used. 10μg proteins were loaded into lanes of barley root and WT, whereas 2μg proteins were used for the lanes of Ox*AlaAT* lines.

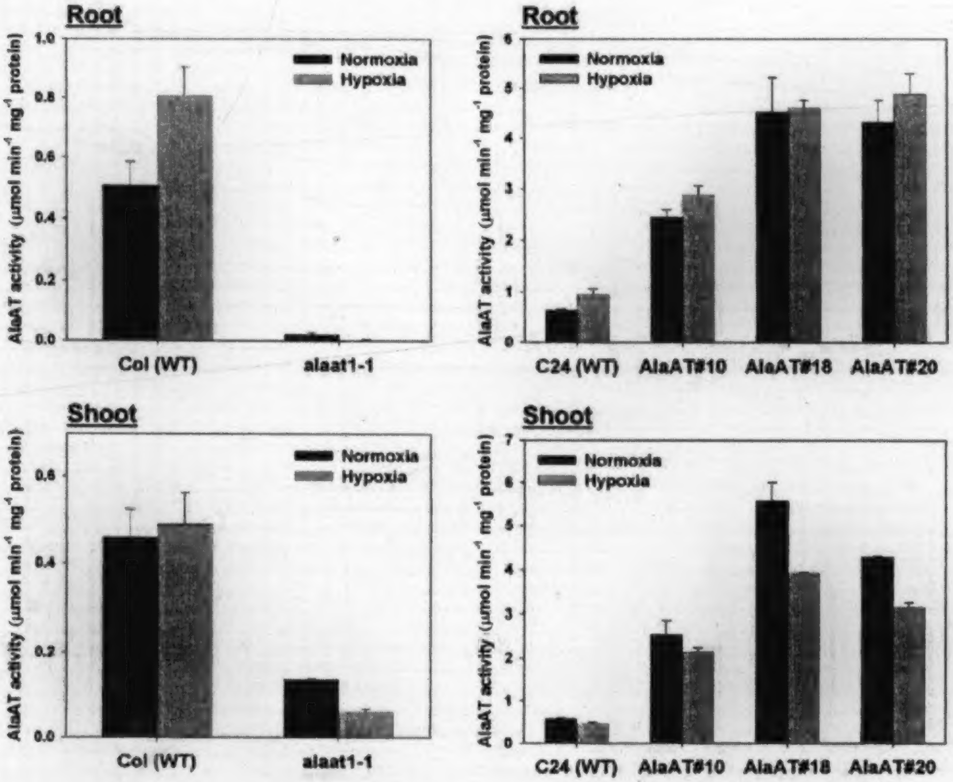


Figure 2.6. AlaAT activity in *alaat1-1*and *AlaAT* over-expressing lines.

17-day-old plants that were treated for hypoxia (5% O_2 for 24 h) and untreated plants were used. AlaAT activity was measured spectrophotometrically using the enzyme extracts of roots and shoots. Results shown are the means of triplicate samples. The error bars represent S.E. The experiments were repeated with similar results.

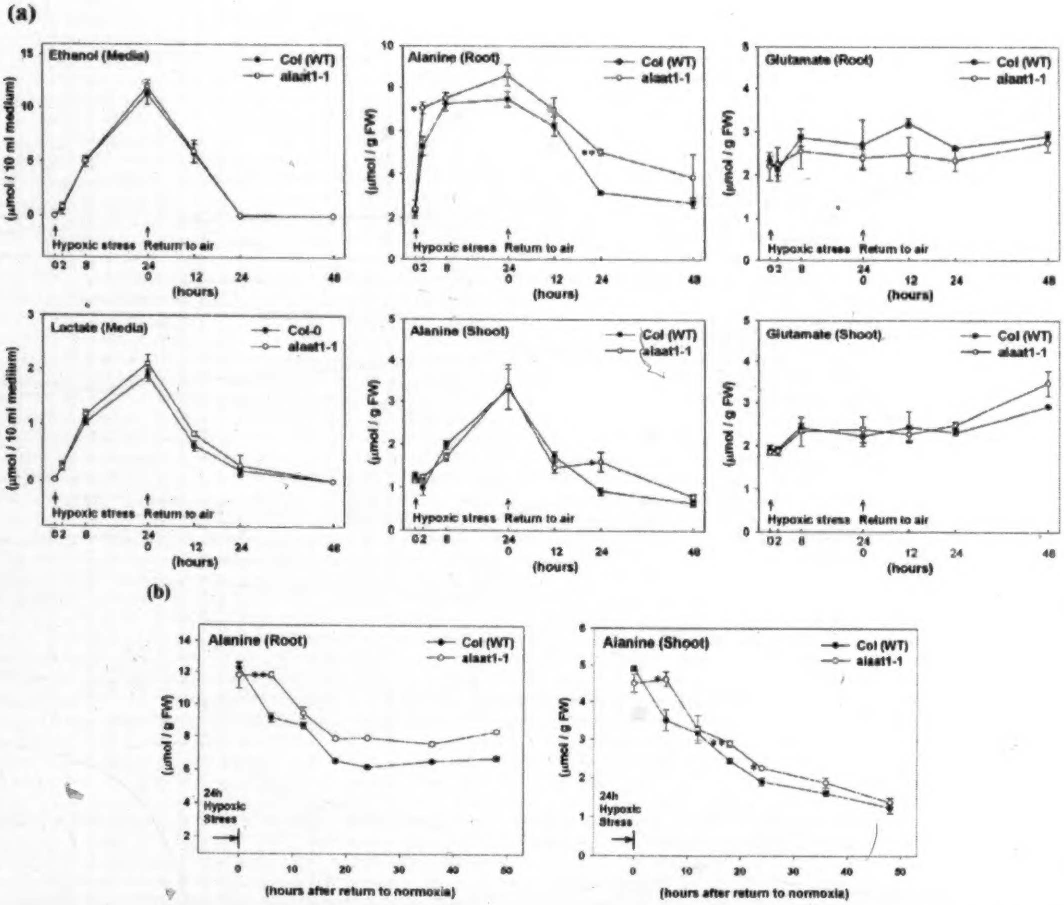


Figure 2.7. Metabolic profiles of *alaat1-1* over the period of hypoxic treatment and the subsequent recovery.

(a) Changes in the metabolites during over the period of hypoxia and post-hypoxia. Plants were subjected for hypoxia (5% O_2) and the metabolites were extracted from the plants harvested at 4 time points during the stress treatment (0 h, 2 h, 8 h, and 24 h). After 24 h of treatment, hypoxically treated plants were returned to normoxic conditions, and the samples were taken at 3 time points (12 h, 24 h and 48 h). (b) Delayed decline in alanine concentration in *alaat1-1* during post-hypoxia. Plants were first hypoxically treated for 24 h, and samples were taken from 7 time points over 48 h recovery periods to analyze the alanine decrease. The level of each metabolite was enzymatically determined. Results shown are the means of triplicate samples. The error bars represent S.E. * and ** indicates P<0.05 and P<0.01 (Student's t-test), respectively. The experiments were repeated with similar results.

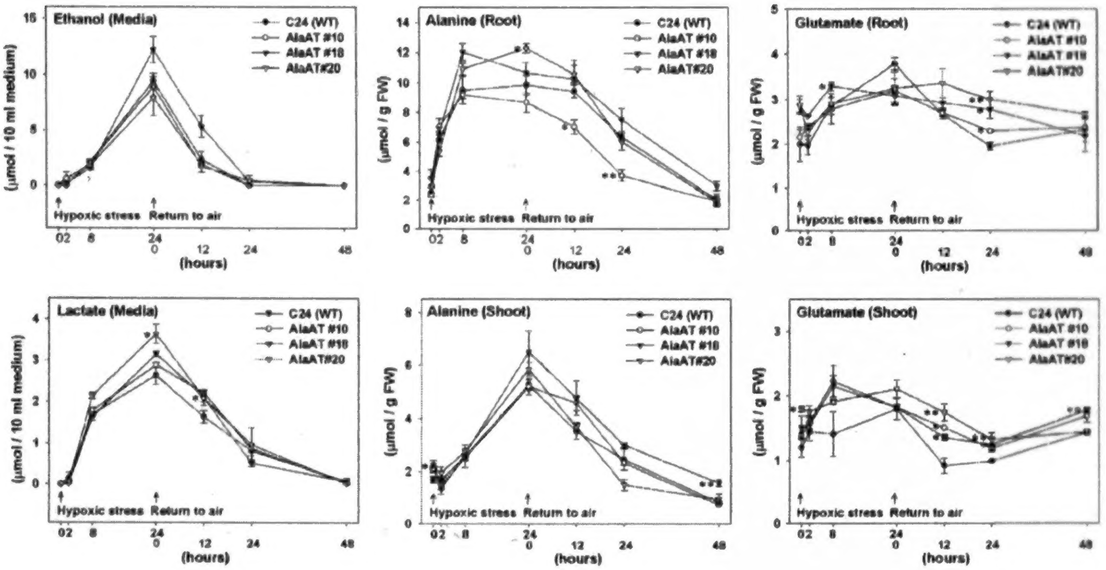


Figure 2.8. Metabolic profiles of Ox*AlaAT* lines over the period of hypoxic treatment and the subsequent recovery.

Plants were subjected for hypoxia (5% O_2) and the metabolites were extracted from the plants harvested at 4 time points during the stress treatment (0 h, 2 h, 8 h, and 24 h). After 24 h of treatment, hypoxically treated plants were returned to normoxic conditions, and the samples were taken at 3 time points (12 h, 24 h and 48 h). The level of each metabolite was enzymatically determined. Results shown are the means of triplicate samples. The error bars are S.E. * and ** indicates $P<0.05$ and $P<0.01$ (Student's t-test), respectively. The experiments were repeated with similar results.

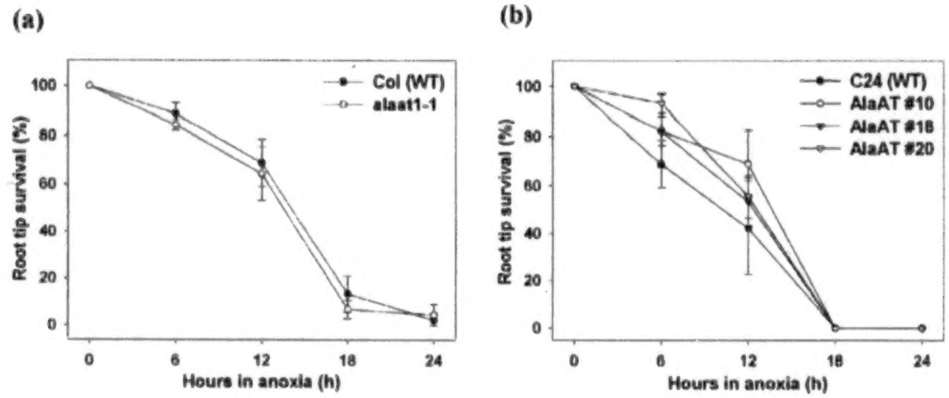
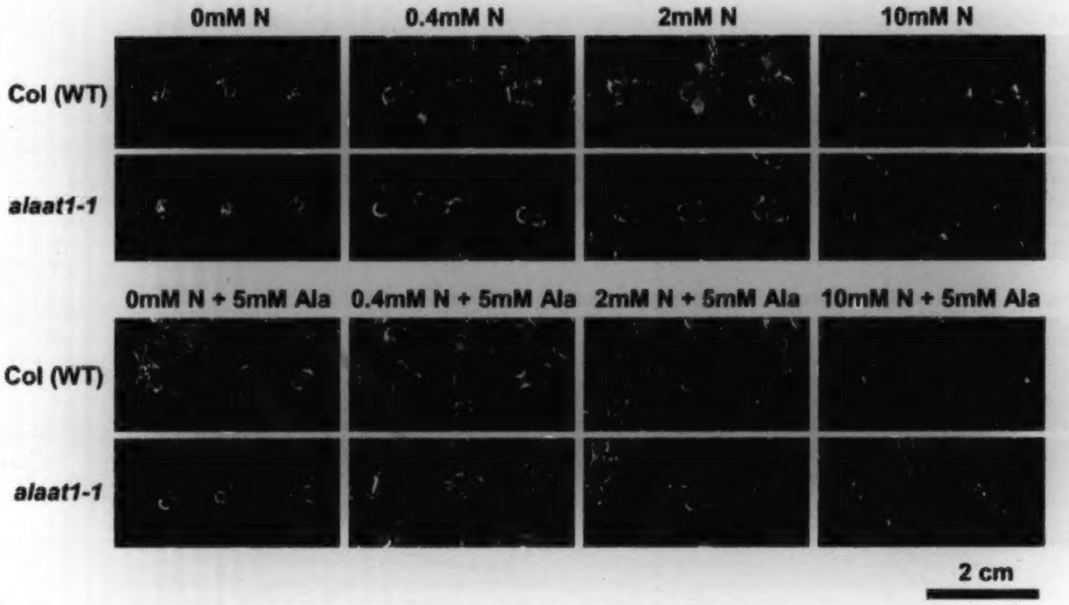


Figure 2.9. Root tip survival under anoxic conditions.

7-day-old seedlings grown on vertical plates were subjected to various lengths of anoxic conditions (full N_2, for 0 h-24 h) in liquid media. The stressed seedlings were then transferred to new vertical plates and allowed to recover. The root tip survival was scored based on the ability of the primary root tip to grow further at 2 days after the transfer. (a) Root tip survival rate of *alaat1-1* and WT. (b) Root tip survival rate of Ox*AlaAT* lines and WT. Results shown are the means of 3 replicate samples. The error bars are S.E. The experiments were repeated with similar results.



0mM N
0.4mM N
2mM N
10mM N
Col (WT)
alaat1-1
0mM N + 5mM Ala
0.4mM N + 5mM Ala
2mM N + 5mM Ala
10mM N + 5mM Ala
Col (WT)
alaat1-1
2 cm

Figure 2.10. Reduced growth of *alaat1-1* on alanine supplementation media.

Plants were grown for 2 weeks on nitrogen-controlled 0.5x MS media with or without additional 5mM alanine supplementation.

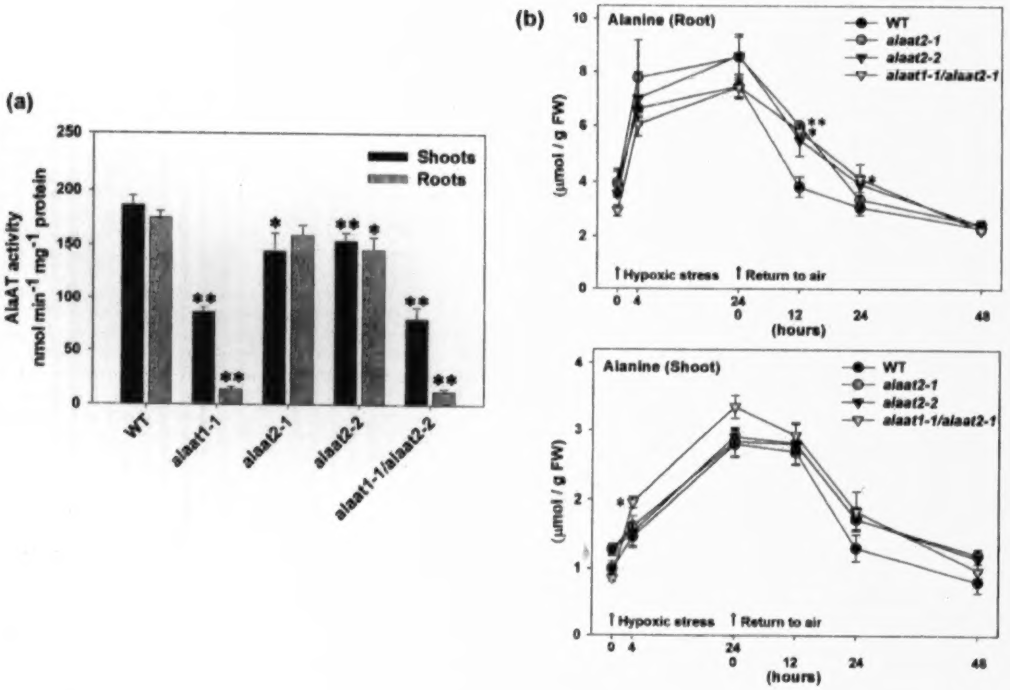


Figure 2.11. Characterization of putative *alaat2* and *alaat1-1/alaat2*-1 double mutants.

(a) Reduced AlaAT activity in the *alaat* mutants. 17-day-old plants were used. AlaAT activity was measured spectrophotometrically using the enzyme extracts of roots and shoots. Results shown are the means of triplicate samples. The error bars represent S.E. (b) Changes in alanine content during hypoxia and the subsequent recovery periods. Plants were subjected for hypoxia (5% O_2) and the metabolites were extracted from the plants harvested at 3 time points during the stress treatment (0 h, 24h and 24 h). After 24 h of treatment, hypoxically treated plants were returned to normoxic conditions, and the samples were taken at 3 time points (12 h, 24 h and 48 h). The levels of alanine metabolite were enzymatically determined. Results shown are the means of triplicate samples. The error bars are S.E. * and ** indicates P<0.05 and P<0.01 (Student's t-test), respectively.

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Appendix 2.1. List of the putative *AlaAT2* mutants identified by TILLING.

Identification of the TILLING mutants were performed by Dr. Erin Gilchrist and Dr. George Haughn at University of British Columbia (The Canadian TILLING Initiative).

Variant No.	Nucleotide Change	Effect	ABRC Seed Stock Number	RE Gained in Variant	RE Lost from Reference	PSSM Difference	SIFT Score	Zygosity
1	G1504A	S148N	CS92024		HphI			Het
2	G1793A	A185T	CS90073	BsrI	AluI,BbvI, CviJI,Fnu4HI, NspBII,PvuII, TseI			Hom
3	G1802A	E188K	CS92461		TaqI			Hom
4	G1846A	L202=	CS92337	MseI	Hpy178III			Hom
5	C1931T	Intron	CS87941	MseI	DdeI			Hom
6	C2155T	L251F	CS87856		Hpy178III			Het
7	C2179T	L259=	CS94707		BsrI,FinI			Het
8	C2189T	S262F	CS88292	SspI	Hpy188I			Hom
9	G2398A	K307=	CS90338		Cac8I			Hom
10	G2653A	R366K	CS89794		BseRI,MnlI	12	0.08	Hom

3. Contribution of the GABA shunt to hypoxia-induced alanine accumulation in roots of *Arabidopsis thaliana*.

3.1. Introduction

When soil is flooded for a prolonged period, plant roots suffer from the depletion of soil oxygen, causing plants to face several challenges for survival. Root damage incurred by oxygen deficiency is mainly due to the inhibition of oxidative phosphorylation and cytoplasmic acidosis (Drew, 1997; Vartapetian and Jackson, 1997). To cope with these problems, plants induce the expression of anaerobic polypeptides (ANPs) and initiate ethanol and lactate fermentation to maintain ATP synthesis under oxygen limiting conditions (Sachs *et al.*, 1980, 1996). The accumulation of certain amino acids is another well-known response of plants to low-oxygen stress (Reggiani *et al.*, 2003). Alanine and γ-aminobutyric acid (GABA) are the two major amino acids synthesized under hypoxic stress conditions and can account for a significant portion of the free amino acid pool in hypoxically stressed tissues (Streeter and Thompson, 1972; Reggiani *et al.*, 1988; Aurisano *et al.*, 1995a; Fan *et al.*, 1997; Sato *et al.*, 2002; Sousa and Sodek, 2003). Therefore, it has been suggested that the accumulation of amino acids is a defence mechanism against oxygen deficiency.

In the study presented here, the 5 homologous *GAD* genes and *GABA-T1* in *Arabidopsis* were studied to elucidate the function of the GABA metabolism under oxygen limiting conditions. Furthermore, we isolated and characterized *Arabidopsis GAD1* and *GABA-T1* mutants to investigate the roles of these enzymes under oxygen deficient conditions and to assess the potential for the GABA-dependent alanine production under hypoxia (see Figure 3.1 for the GABA shunt pathway in *Arabidopsis*). The inhibitory effects on the hypoxia-induced alanine accumulation observed in these mutants demonstrated that the operation of the GABA shunt contributes to alanine accumulation in the roots of *Arabidopsis*.

NOTE: *A version of this chapter has been published. Miyashita, Y. and Good, A.G. (2008) Plant and Cell Physiology. 49: 92-102.*

3.2. Materials and Methods

3.2.1. Plant materials and plant growth conditions

Arabidopsis plants of Columbia-0 (Col-0), Landsberg erecta (L. er) and Wassilewskija (Ws) ecotypes were used in this study. The Salk T-DNA mutant lines (ecotype: Col-0), Salk_007661 (*gaba-t1-1*), Salk_047648 (*gad1-4*), Salk_082173 (*gad1-5*), Salk_022227 (*gad1-6*), and the previously characterized GABA-T deficient mutants, *pop2-1* (ecotype: L. er) and *pop2-3* (ecotype: Ws) (Palanivelu *et al*. 2003) were obtained from Arabidopsis Biological Resource Center (ABRC). The mutant plants homozygous for the T-DNA insertions were isolated from the obtained seed stocks using the PCR screening scheme described at Salk institute web page (http://signal.salk.edu.).

Arabidopsis plants were typically grown in potting mixture, MetroMix 220 (Scotts Co, Marysville, OH, USA), or in 0.5X MS media (1% sucrose) at 22-23 °C and at approximately 150 µmol m^{-2} sec^{-1} light on a 16-h light/ 8-h dark cycles unless otherwise indicated. The soil-grown 40-day-old plants were used to harvest flower, silique, stem, and rosette leaf tissues for the gene expression study.

In order to collect shoot and root tissues, *Arabidopsis* plants were also grown semihydroponically as described previously (Miyashita *et al*., 2007). Briefly, seeds were surface-sterilized with full-strength commercial bleach for 10 min and sown on nylon mesh which was placed on top of 0.5X MS media (1% sucrose and 0.4 % agar). After 2 days of stratification at 4 °C, seeds were allowed to germinate and to grow for 10 days under the standard growth conditions as described above. These 10-day-old seedlings growing on nylon mesh were then transferred to liquid 0.5X MS media (1% sucrose) for an additional 7 days of growth. The shoot and root tissues harvested from these 17-day-old plants were used for RNA, protein and metabolite extractions. To assess the ability of *Arabidopsis* plants to utilize GABA as a nitrogen source, plants were grown on nitrogen-free 0.5X MS medium (1.0% sucrose and 0.6% agar) supplemented with 10 mM GABA. Typically, seeds of *gaba-t1* mutant lines were co-plated with their corresponding WT lines. Pictures of the plants growing on GABA-supplemented plants were taken after 2 weeks of growth under the standard conditions described above.

3.2.2. Hypoxic treatment and anoxia survival test

For the hypoxic treatment, 17-day-old plants grown semihydroponically were used. The hypoxic treatments were carried out as described previously (Miyashita *et al.*, 2007). Briefly, the liquid 0.5X MS medium on which plants were grown for 7 days was replaced with new liquid 0.5X MS medium that had been gassed with 5% oxygen gas (balanced with nitrogen) for 15 min. After exchanging the liquid medium, plates were stacked in anaerobic jars. The anaerobic jars were then flushed with 5% oxygen gas for additional 15 min and left in the dark for the duration of the treatments (for analysis of the transcriptional induction; 2 h, 8 h and 24h, for metabolite assays; 4h, 24 h and 48 h). In addition, untreated plants were used as the 0 h hypoxia control. The shoot and root tissues harvested from the untreated and hypoxically treated plants were flash frozen in liquid nitrogen and used to measure the changes in the levels of transcripts, enzyme activities and metabolites. Also, as a control experiment, plants kept in the dark under aerobic conditions for the same duration as the hypoxic treatments were used to demonstrate the changes in the metabolites were caused by the hypoxic treatments. Also, the anoxia survival test was performed as described previously (Ellis *et al.*, 1999) to assess the anaerobic tolerance of the mutants and their corresponding WT lines.

3.2.3. RT-PCR and quantitative real time RT-PCR analyses

RNA extraction, DNase treatment, and first-strand cDNA synthesis were performed as described (Miyashita *et al.*, 2007). For the RT-PCR analysis to demonstrate the absence of the corresponding transcripts in *gaba-t1* and *gad1* mutant lines, amplifications were performed with the synthesized cDNA as templates and with the following gene-specific primers; *GABA-T1*, 5'-GCATGTCTGGGAACTCCACT-3' and 5'-AGGCAAACGCTTTAACTCCA-3', *GAD1*, 5'- TTCAAGATGCCGGAAAACTC-3' and 5'-TCCTTCCCTTAGGACGATCA-3' and *β-Tubulin* (see Kang and Singh 2000).

For quantitative real time RT-PCR, PCR reaction and the SYBR Green detection were performed on a light cycler ABI Prism 7000 (Applied Biosystems). Each reaction was performed in triplicate, and the specific amplification of the target was checked with a heat dissociation protocol (60 °C to 95 °C) after the completion of the PCR reaction. Gene-specific primers for real time RT-PCR were designed to amplify 125-150 bp sequences found in the coding regions near the 3'-end of each gene; *GABA-T1*, 5'-

CAGGTGATGGCATTTTGATG-3' and 5'-ACTTCTTGTGCTGAGCCTTGA-3'; *GAD1*, 5'-TTCTTCACCGTGACCATCAA-3' and 5'-CGCTGAGAGACTTGTGATCG-3'; *GAD2*, 5'-CCATCAGAATCTCCTTCTCCA -3' and 5'-ACACTCGCGGAGAGACTTGT-3'; *GAD3*, 5'-GCCAAGATGGCTAGTGGAAA-3' and 5'-GGTAATACTTGCTACTAACGGAACG-3'; *GAD4*, 5'-GTTCACGCCAAGATGGCTAA-3' and 5'-GCAAATTGTGTTCTTGTTGGTC-3'; *GAD5*, 5'-CGAGGCCTTGCAGATAGACT-3' and 5'-GGACATCTTGGCAGTCTTCAC-3'; *ACT2*, 5'-TATCGCTGACCGTATGAGCA-3' and 5'-TTACCTGCTGGAATGTGCTG-3'. The amplified fragments were cloned into pCR2.1-TOPO vector (Invitrogen). To quantify the transcripts of each gene, the copy numbers of each target were determined by the standard curve constructed using the diluted plasmid samples. The determined copy numbers of each transcript were then normalized against the copy numbers of *ACT2* transcripts. The transcript abundance of different genes (e.g. among the 5 homologous *GAD* genes) was indirectly compared by using the copy number of each target transcript normalized as above.

3.2.4. Enzyme assays

For GABA-T assay, protein extraction was carried out in an extraction buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1.5 mM DTT, 100 μM PMSF, 10 μg ml^{-1} Luepeptin, 10% (v/v) glycerol. 3X vol and 4X vol of the extraction buffer were added to shoot and root tissues, respectively, and the tissues were ground using ice-cold mortar and pestle with a pinch of sand and PVPP. After grinding the tissues, samples were centrifuged at 16 000 g for 10 min to remove the cell debris, and the supernatant was used for the enzyme assay. The GABA-T activity was measured in a direction toward alanine/SSA or glutamate/SSA production by quantifying the amount of alanine or glutamate synthesized as described (Ansari *et al.*, 2005). In short, using a small volume of the crude enzyme extracts (typically 25 μl), the GABA-T reaction was allowed to proceed in the assay mix containing 50 mM Tris-HCl (pH 8.0), 1.5 mM DTT, 0.75 mM EDTA, 0.1 mM pyridoxal-5-phosphate (PLP), 10% (v/v) glycerol, 16 mM GABA, and 4 mM of either pyruvate or 2-oxoglutarate in a final volume of 500 μl. After the incubation at 30 °C for 1 h, sulfosalicylic acid was added to a final concentration of 4 mM to stop the reaction. To calculate GABA:pyruvate transaminase and GABA:2-oxoglutarate transaminase activities, the amount of alanine or glutamate synthesized was measured by enzyme-linked assays with alanine dehydrogenase (AlaDH) or glutamate dehydrogenase (GDH). AlaDH assay was performed in the assay mix with 150 mM Tris-HCl (pH 9.0), 1.0 mM NAD^+ and 0.02 U of *Bacillus subtilis* AlaDH (Sigma) on a 96-well plate. The increase in OD_{340} was measured using the 96-well ELISA plate reader (SpectroMAX+) (Molecular Devices, Sunnyvale, CA, USA), and the amount of the synthesized alanine was determined by comparison to the samples containing the known concentrations of alanine. The GDH assay was done in the assay mix with 150 mM Tris-acetate (pH 8.5), 1.0 mM NAD^+, 0.2 mM ADP and 1.5 U of GDH on a 96-well plate. The increase in OD_{340} was measured using the 96-well ELISA plate reader, and the amount of the synthesized glutamate was determined by comparison to the samples containing the known concentrations of glutamate.

For the GAD assay, enzyme extraction was carried out in an extraction buffer containing 100 mM Tris-HCl (pH7.5), 1 mM EDTA, 1 mM PMSF, 10% (v/v) glycerol. 3X vol and 6X vol of the extraction buffer were added to shoot and root tissues, respectively. Grinding and centrifugation was performed as described above to prepare the crude enzyme extracts. The GAD reaction was allowed to proceed with a small volume of the enzyme extracts (typically 20 µl) in the assay mix with 150 mM potassium phosphate (pH 5.8), 0.1 mM PLP and 20 mM glutamate in a final volume of 200 µl. After the incubation at 30 °C for 1 h, perchloric acid was added to a final concentration of 0.6 M to stop the reaction, and the samples were neutralized immediately with 3 M KOH. To calculate GAD activity, the amount of GABA in the neutralized samples was measured by an enzyme-linked assay using *Pseudomonas fluorescens* GABase (Sigma, St Louis, MO, USA) that catalyzes the conversion of GABA to SSA and then to succinate with a stoichiometric reduction of $NADP^+$. The GABase assay was performed in the assay mix with 75 mM potassium pyrophosphate (pH 8.6), 3.3 mM β-mercaptoethanol, 10 mM 2-oxoglutarate, 1.25 mM $NADP^+$, 0.016 U of GABase that had been dissolved in 75 mM potassium phosphate buffer with 25% (v/v) glycerol (pH 7.2) immediately before use. The increase in OD_{340} was measured using the 96-well ELISA plate reader, and the amount of the synthesized GABA was determined by comparison to the samples containing the known concentrations of GABA.

3.2.5. Metabolites assays

Metabolite extractions were performed as described previously (Good and Muench, 1993). Harvested plant tissues were ground in 0.6 M perchloric acid using ice-cold mortar and pestle with a pinch of sand. The perchloric acid extracts were neutralized with 3 M KOH and stored at –80 °C until used for the metabolite assays. The levels of GABA, alanine and glutamate were measured by GABase, AlaDH and GDH assays, respectively, as described above. Subsets of samples were also spiked with known amounts of metabolites to check the validity of the assays.

3.3. Results

3.3.1. *GAD* and *GABA-T* genes and their expression in *Arabidopsis*.

In *Arabidopsis*, two cDNAs encoding GAD (*GAD1*: At5g17330, *GAD2*: At1g65960) have been cloned and characterized previously (Turano *et al*., 1998). Subsequently, the whole-genome search for other *GAD* genes has identified 3 other putative *GAD* genes (*GAD3*: At2g02000, *GAD4*: At2g02010, *GAD5*: At3g17760), constituting a small gene family of 5 *Arabidopsis GAD* homologues (Shelp *et al*. 1999). For GABA-T, only one gene (*GABA-T1*: At3g22200) has been identified in *Arabidopsis* (Van Cauwenberghe *et al*., 2002). For the specificity of the transamination catalyzed by GABA-T, the recombinant *Arabidopsis* GABA-T1 expressed in *E. coli* showed catalytic activity with only pyruvate, although both pyruvate- and 2-oxoglutarate-dependent GABA-T activities have been detected in plants (Van Cauwenberghe *et al*., 2002). In *Arabidopsis*, 2-oxoglutarate-dependent GABA-T activity was not detected (data not shown, Van Cauwenberghe *et al*., 2002), so the identity of the enzyme catalyzing the 2-oxoglutarate-dependent GABA-T reaction is unclear.

In the present study, we analyzed the expression of the genes encoding GABA-T and GAD in various organs of *Arabidopsis* using quantitative real time RT-PCR (Figure 3.2). For *GABA-T1*, the transcript of *GABA-T1* was detected at varying levels in all organs tested, with somewhat higher levels of expression in flowers, siliques and entire shoots of young seedlings than in the other organs (Figure 3.2A). Of the *GAD* genes, *GAD2* expression was nearly constitutive at relatively high levels in all organs tested except for the slightly lower expression in sliques, whereas *GAD1* expression was predominantly found in roots with lower expression in shoots of young seedlings and minuscule expression in the other organs (Figure 3.2B). The expression of the other 3 *GAD* genes was generally weak in all organs except for the expression of *GAD4* and *GAD5* in flowers (Figure 3.2B). The observed expression patterns for *GAD1* and *GAD2* were similar to previous expression studies which showed the root-specific expression of *GAD1* and the strong expression of *GAD2* in roots, young leaves and flowers but not in siliques (Turano *et al.*, 1998; Zik *et al.*, 1998; Bouché *et al.*, 2004). In addition, AtGenExpress microarray data (Schmid *et al.*, 2005) generally support our results for the expression of the 5 *GAD* genes (data not shown). Based on this expression data, it appears that *GAD1* and *GAD2* are the highest expressed genes among the 5 *Arabidopsis GAD* homologues under normal growth conditions.

3.3.2. Transcriptional modulation in response to hypoxia

To study the induction of *GAD* and *GABA-T1* genes under low-oxygen stress conditions, we monitored the expression of these genes by quantitative real time RT-PCR using the total RNA extracted from roots of 17-day-old plants treated for various periods of hypoxia (5% O_2). For the control of the experiment, the expression of a well-characterized hypoxia-inducible gene, *ADH1* (Dolferus *et al.*, 1994), was also analyzed. Results showed the rapid induction of *ADH1* expression under hypoxic conditions (Figure. 3.3A), and this confirmed that the plant samples used in this experiment were experiencing hypoxia. In contrast, the expression of *GABA-T1* was not significantly affected by the hypoxic treatment (Figure 3.3B). It appeared that there was a slight decrease in *GABA-T1* expression at 2 h after the onset of the stress; however, this decrease was not statistically significant due to the large variance found in the untreated control (0 h). For the homologous 5 *GAD* genes, different responses of the expression of the 5 *GAD* genes to the hypoxic conditions were observed. For instance, *GAD2* expression was gradually repressed during the stress treatment (Figure 3.3C). This repression did not occur during the initial phase of the hypoxia but became evident at the subsequent time points in which the transcripts levels were diminished to approximately ½ and 1/4 of the level compared to the untreated control after 8 h and 24 h of hypoxia, respectively (Figure 3.3C). Conversely, the expression of *GAD4* was found to be highly inducible by the hypoxic stress treatment. In fact, *GAD4* expression increased approximately 3-, 19- and 13-fold after 2, 8 and 24 h of hypoxia, respectively (Figure 3.3C). Thus, *GAD4* appeared to be the most highly expressed gene among the 5 *GAD* homologues after 8 h and 24 h of the stress treatment. As to *GAD1*, the expression of *GAD1* was maintained at the level similar to the untreated control throughout the hypoxic treatment (Figure 3.3C). The transcripts of the other two *GAD* genes, *GAD3* and *GAD5*, were nearly undetectable throughout the time-course of this experiment (Figure 3.3C).

3.3.3. Isolation and characterization of *Arabidopsis gaba-t1* and *gad1* mutants

The Salk T-DNA insertion lines (Alonso *et al.*, 2003) were obtained from the ABRC and screened for *gaba-t1* and *gad1* mutants. The screen resulted in the isolation of one mutant (*gaba-t1-1*) containing T-DNA in *GABA-T1* and 3 mutants each containing T-DNA in *GAD1* (Figure 3.4A, 3.4C; *gad1* mutants identified in this study were designated as *gad1-4*, *gad1-5*, *gad1-6* as there has been a report of other *gad1* mutants, see Bouché *et al.*, 2004). The sites of the T-DNA insertions in each mutant were verified by sequencing the PCR products flanking the insertion junctions (data not shown). The previously characterized GABA-T mutants, *pop2-1* and *pop2-3* (Palanivelu *et al.*, 2003), were also used in this study. RT-PCR analysis showed the absence of the functional transcripts of *GABA-T1* and *GAD1* in each corresponding mutant except for *pop2-1* that carries a point substitution (Figure 3.4B, 3.4D). Reduction in the enzyme activity was also confirmed by assaying the activity of the corresponding enzymes in roots and shoots of 17-day-old plants grown on liquid 0.5X MS media. For GABA-T, the activity was reduced to nearly negligible levels in each mutant in both shoots and roots (Figure 3.5A). Moreover, reduction of GABA-T activity to negligible levels in the *gaba-t1* mutants was demonstrated by the inability of these mutants to grow on the media containing GABA as the sole nitrogen source (Figure 3.6). This indicated that the mutants cannot mobilize and utilize the amino N of GABA for growth. GAD activity in the roots of *gad1* mutants was reduced to 20-25 % of the activity found in the roots of WT plants, while the reduction in the shoots of the mutants was not significant (Figure 3.5B). These results indicate that the GAD activity in the roots of *Arabidopsis* results largely from *GAD1* expression. Considering the strong expression of *GAD2* and very weak expression of the other 3 *GAD* genes (Figure 3.2B), the remaining activity is presumably due to GAD2. Also, *GAD2* expression seems to be responsible for most of the activity found in the shoots of *Arabidopsis*. This is supported by the weak expression of the other 4 *GAD* genes in shoot tissues and by the similar GAD activity found in the shoots of *gad1* mutants and WT plants (Figure 3.5B). The clear reduction in GAD activity in the roots of *gad1* mutants allowed us to study the role of GAD in the roots of *Arabidopsis*.

In terms of the phenotypes of the *gaba-t1* and *gad1* mutants, we did not observe any abnormalities in growth and development when these mutants were grown on soil and on 0.5X MS media under normal growth conditions, except for the reduced seed production in the *pop2* mutants as described previously (Palanivelu *et al*., 2003). In order to investigate whether the defect in GABA metabolism affects the tolerance of plants to oxygen deficiency, we subjected 7-day-old seedlings to anoxic conditions for up to 36 h and scored the survival of the treated seedlings. In one experiment, the *gad1* mutants looked more susceptible to anoxic conditions, however this phenotype could not be repeated in two other independent experiments (data not shown). The *gaba-t1* mutants behaved much like WT plants under anoxic stress. Thus, the role of GABA and its metabolism in anaerobic stress tolerance was not demonstrated in this study.

3.3.4. Changes in metabolite levels during hypoxic stress conditions

To study the function of GABA-T and GAD under low-oxygen conditions, we treated 17-day-old plants to various time periods of hypoxia (5% O_2) and compared the levels of metabolites in *gaba-t1* and *gad1* mutants to those in the corresponding WT lines. The levels of GABA in the roots of *gaba-t1* mutants were invariably higher than those in roots of WT plants, and GABA accumulation during the hypoxic treatments was observed in *pop2-1* mutant but not in the corresponding WT plants (Figure. 3.7). The elevation of GABA levels was also detected in the shoots of *gaba-t1* mutants during hypoxic treatments (Figure 3.7). The increase of GABA in the *gaba-t1* mutants can be explained by the inhibition of the conversion of GABA to SSA as the result of the loss of GABA-T activity in *gaba-t1* mutants. A similar metabolic phenotype has been previously observed in *pop2* mutants where the GABA contents in leaves were several-fold higher than those in the leaves of WT plants (Palanivelu *et al*., 2003). In addition, alanine accumulation occurred in the roots of both *gaba-t1* mutants and WT plants at a similar rate during the initial phase of hypoxia, however, the levels of accumulated alanine were lower in *gaba-t1-1* and *pop2* mutants than those in corresponding WT plants at 24 h after the onset of the hypoxic treatments (Figure 3.7). In shoots, alanine accumulation occurred with no difference in the levels of the accumulated alanine between *gaba-t1* mutants and WT plants (Figure 3.7). These data suggest that GABA-dependent alanine production catalyzed by GABA-T contributes to the hypoxia-induced alanine accumulation in the roots but not in the shoots of *Arabidopsis*.

For *gad1* mutants, reduced GABA levels and elevated glutamate levels were detected in the roots of *gad1* mutants compared to the levels of these metabolites in WT plants (Figure 3.8). This metabolic phenotype in *gad1* mutants was similar to that of the previously characterized *gad1* mutants (Bouché *et al.* 2004). Moreover, the alanine accumulation under hypoxia was reduced in the roots of *gad1* mutants compared to that in WT plants at 24 h after the onset of the hypoxic treatments (Figure 3.8). In the shoots of *gad1* mutants, the levels of metabolites were generally similar to those in the shoots of WT plants (Figure 3.8). Therefore, the metabolite analysis on *gad1* mutants supports the conclusion that GABA metabolism is operative and contributes to alanine production in roots but not in shoots under low-oxygen conditions. However, the GABA-T reaction appears to be responsible for only a small portion of alanine synthesized in roots during hypoxic conditions, and other mechanism(s) are likely responsible for the rapid alanine accumulation in roots during the initial phase of hypoxia and for the alanine accumulation in shoots.

As a control experiment, we measured the metabolite levels in the plants kept in the dark under aerobic conditions since the hypoxic treatments were done in the dark (to avoid a generation of oxygen by photosynthesis). The results showed no significant changes in the levels of metabolites under the dark aerobic conditions except the increase of alanine observed in the shoots of *pop2-3* and its corresponding WT plants (data not shown). These results demonstrate that the observed changes in the metabolite levels are mainly due to the exposure of the plants to hypoxic stress conditions. Also, the different metabolite levels and metabolic changes among the *Arabidopsis* lines of distinct backgrounds observed in this study are consistent with previous studies showing differences in the metabolic profiles in different ecotypes (Fiehn *et al.*, 2000; Bouché *et al.*, 2004).

3.4. Discussion

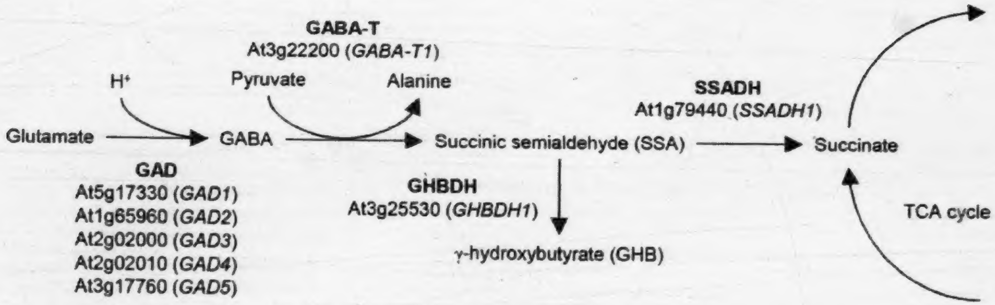
To investigate the function of GABA metabolism under low-oxygen stress conditions, we have studied the expression of the genes encoding the enzymes of the GABA shunt, GABA-T and GAD, and characterized the mutants defective in these genes. The focus of this study was to examine GABA metabolism under hypoxic conditions and to re-assess the possibility of the hypoxically induced synthesis of alanine through the GABA-T reaction by analyzing *gaba-t1* and *gad1* mutants.

To study the function of the GABA shunt, we measured the transcript levels of *GABA-T1* and the 5 homologous *GAD* genes in various organs of *Arabidopsis*. Moreover, the changes in the transcript levels of these genes under hypoxic conditions were monitored in the roots of the hypoxically stressed plants. For *GABA-T1*, its expression was detected in all organs, and its expression was slightly repressed during hypoxic treatments (Figure 3.2A, 3.3B). This expression pattern was similar to that seen in the AtGenExpress microarray data (Schmid *et al.*, 2005) and the microarray data prepared from hypoxically treated plants (Loreti *et al.*, 2005). Since *GABA-T1* expression was detected in virtually all organs/tissues, the GABA-T activity seems to be universally present in plant tissues. However, the similar levels of GABA-T activity in both roots and shoots of WT lines (Figure 3.5A) suggests the preferential translation of *GABA-T1* transcripts or the increased stability of GABA-T protein in roots because *GABA-T1* transcript levels were significantly higher in shoots than in roots (Figure 3.2A). For the involvement of *GABA-T* in other aspects of plant development, the induction of the rice *GABA-T* gene, *Osl2*, was observed in senescing leaves, indicating the potential role of the GABA shunt in C/N metabolism during the senescence process (Ansari *et al.* 2005). Supporting this role of GABA metabolism during senescence, the up-regulation of *Arabidopsis GABA-T1* during senescence was also found using the Genvestigator Response Viewer (Zimmermann *et al.*, 2004). For the expression of the *GAD* genes, quantitative real time RT-PCR allowed us to separately measure the abundance of each *GAD* transcript, as quantifying the expression of genes that exist in multiple copies in a genome is often difficult by hybridization-based tests. It should be noted that microarray analyses using the Affymetrix ATH1 array were not able to distinguish the transcripts of

GAD3 and *GAD4* due to the high sequence identity between the two transcripts, and thus identical expression values were given for both *GAD3* and *GAD4*. In the present study, we have confirmed the predominant expression of *GAD1* in roots and the nearly constitutive expression of *GAD2* as previously described (Figure 3.2B, Turano *et al.*, 1998; Zik *et al.*, 1998; Bouché *et al.*, 2004). The expression of *GAD1* in roots was also confirmed by analyzing the expression of reporter genes under the control of *GAD1* promoter (Bouché *et al.*, 2004). For the other 3 *GAD* genes, the expression of *GAD4* and *GAD5* were detected only in flowers, and that of *GAD3* was negligible in all tissues tested (Figure 3.2B). In response to hypoxic stress conditions, up-regulation was observed for *GAD4* only (Figure 3.3C). In the previously conducted microarray experiments investigating the transcriptional changes during low-oxygen conditions, this strong induction of *GAD4* was not observed (Liu *et al.*, 2005; Loreti *et al.*, 2005; Branco-Prince *et al.*, 2005). In these microarray experiments, RNA samples were prepared from whole seedlings, whereas we used RNA extracted from the roots of hypoxically treated plants since roots are the tissues most frequently subjected to low-oxygen stress in natural environment. Whether the difference in the samples analysed caused the observed discrepancy in the induction of *GAD4* remains uncertain. Also, interesting induction patterns for *GAD3* and/or *GAD4* (not distinguished by the microarray) were found by the Genvestigator Response Viewer (Zimmermann *et al.*, 2003), where the expression of these genes was shown to be highly up-regulated by several biotic and abiotic stresses such as treatments with *Botrytis cinerea*, $AgNO_3$, cycloheximide, hydrogen peroxide, ozone, syringolin, salt stress and cold stress. Since the induction of a stress-inducible gene by several different stress treatments is a common phenomenon, *GAD4* appears to be the primary *GAD* gene induced by these stresses. With respect to the induction of GAD activity during the early stage of oxygen deprivation or the temporal anaerobiosis, the biochemical induction of GAD by Ca^{2+}/calmodulin or by decreased cytoplasmic pH (Ling *et al.*, 1994; Aurisano *et al.* 1995b; Snedden *et al.*, 1995; Zik *et al.*, 1998) appears more important than the transcriptional induction of *GAD4* since the biochemical activation of GAD can instantly initiate GABA synthesis upon exposure to low-oxygen stress. Supporting this notion, no increase in GAD activity was detected in both WT and *gad1* mutant plants during hypoxic stress conditions (data not shown). GAD activity in roots of *gad1* mutants remained approximately 20 –25 % of the activity found in roots of WT plants after 24 h of hypoxic treatment. The effect of the up-regulation of *GAD4* on the GAD activity may become important when subjected to more prolonged oxygen deficiency.

In terms of the physiological role of the GABA metabolism under low-oxygen conditions, we could not demonstrate the ameliorating effect of GABA accumulation against oxygen deprivation because both *gaba-t1* and *gad1* mutants did not show increased susceptibility to low-oxygen stress. Nonetheless, the metabolic phenotypes observed in *gaba-t1* mutants indicate that the increased GABA synthesis occurs under hypoxic conditions, although the actual increases in the GABA content in WT lines were small in our experiment. Thus, the involvement of GAD in the regulation of cellular pH appears a plausible hypothesis, but estimating the extent of the contribution of GAD to counteract the fall in cellular pH was not done in this study as it requires the use of the mutants completely lacking GAD activity and a non-destructive monitoring of cellular pH during oxygen deprivation. Furthermore, GABA accumulation may proceed better under strict low-oxygen conditions than relatively moderate hypoxic conditions (5 % O_2 was used in our experiments), since the increases in the GABA content were more evident in both shoots and roots of the anoxically treated plants (data not shown). In terms of the GABA synthesized under low-oxygen conditions, it may accumulate in the stressed tissues or be converted to SSA by GABA-T, transferring its amino group to pyruvate to form alanine. Although it has been suggested that the latter reaction does not proceed readily under oxygen deficient conditions (Streeter and Thompson, 1972), our results provide evidence for the GABA-dependent alanine synthesis in roots under hypoxia. It should be noted that the defect in the GABA shunt pathway (the loss of either GAD or GABA-T) affected the alanine accumulation only in roots but not in shoots of *Arabidopsis*. The increased GABA accumulation in the shoots of *gaba-t1* mutants (*pop2-1* and *gaba-t1-1*) suggests that at least some of the GABA produced in the shoots of the WT plants are actively metabolized by GABA-T under hypoxic conditions (Figure 3.7). However, why the hypoxia-induced alanine accumulation was affected only in roots but not in shoots is not clear at this point. Also, SSA produced by the GABA-T reaction is likely to be converted to GHB under low-oxygen conditions, supported by the detection of GHB in anaerobically stressed plant tissues (Breitkreuz *et al*., 2003; Allan *et al*., 2003). This in turn explains the undetectable levels of SSA in the study conducted by Streeter and Thosmpson (1972). Furthermore, it appears that the hypoxia-induced alanine accumulation in roots can be divided into two phases, the initial rapid build-up (during the first 4 h of hypoxia) and the subsequent slow increase (beyond 4 h of hypoxia). In our experiments, the initial phase of the rapid alanine accumulation was not impaired, only the later phase of the slow accumulation was partially inhibited in *gaba-t1* and the *gad1* mutants (Figure 3.7, 3.8). These results suggest that the GABA shunt is likely responsible for part of the slow phase of the alanine accumulation in roots.

As to the physiological role of the GABA shunt, phenotypes observed in the mutants deficient in the enzymes of the pathway indicate the involvement of GABA metabolism in several physiological and developmental phenomena in plants (Bouché and Fromm, 2004). For instance, characterization of the GABA-T deficient mutant, *pop2*, has revealed the key role of the GABA concentration gradient in pollen tube growth and guidance (Palanivelu *et al*., 2003). In addition, the inability of *gaba-t1* mutants to grow on the GABA-supplemented media demonstrated that plants could utilize GABA as an alternate N source when inorganic N is absent (Figure 3.6). This substantiates the involvement of the GABA shunt in N metabolism and the potential role of GABA as N storage. On the other hand, the loss of SSADH activity resulted in the accumulation of reactive oxygen intermediates (ROI) and the hypersensitivity to environmental stresses such as UV-B light and heat stress (Bouché *et al*., 2003). Interestingly, treating the SSADH-null mutants (*ssadh*) with a specific GABA-T inhibitor prevented the accumulation of ROI and GHB in the *ssadh* mutants, inhibited cell death and improved growth (Fait *et al*., 2005). These studies indicate the essential role of the GABA shunt in preventing ROI accumulation and cell death; however, the phenotype of *ssadh* mutants may depend on the accumulation of certain metabolites of the pathway (GHB or SSA). In contrast, the reduction in GAD activity caused by mutations in *GAD1* resulted in changes in metabolite levels in roots but did not result in any morphological defects or developmental abnormalities (this study, Bouché *et al*., 2004). This can be explained by the potential redundant functions of the homologous *GAD* genes in *Arabidopsis*. Conversely, the expression of the truncated *GAD* lacking the calmodulin-binding domain in tobacco and rice resulted in extremely high levels of GABA and severe growth abnormalities, such as dwarfism and the failure of cortex parenchyma to elongate (Baum *et al*., 1996; Akama and Takaiwa, 2007). As seen in the cases of the previously mentioned mutants and transgenic plants, there seems to be no common phenotype caused by the alteration of the GABA shunt. Since the observed phenotypes may be controlled by changes in the levels of certain metabolites in the pathway (often GABA), a better understanding of the function of each metabolite is essential in interpreting the phenotypes observed in the GABA shunt deficient mutants and elucidating the physiological role of the pathway in plants.



GABA-T
At3g22200 (GABA-T1)
H⁺
Pyruvate
Alanine
Glutamate
GABA
Succinic semialdehyde (SSA)
Succinate
SSADH
At1g79440 (SSADH1)
GAD
At5g17330 (GAD1)
At1g65960 (GAD2)
At2g02000 (GAD3)
At2g02010 (GAD4)
At3g17760 (GAD5)
GHBDH
At3g25530 (GHBDH1)
γ-hydroxybutyrate (GHB)
TCA cycle

Figure 3.1. Schematic representation of the GABA shunt in *Arabidopsis*.

GAD; glutamate decarboxylase, GABA-T; γ-aminobutyric acid transaminase, GHBDH; γ-hydroxybutyrate dehydrogenase, SSADH; succinic semialdehyde dehydrogenase. The locus numbers shown are for the *Arabidopsis* genes encoding the corresponding enzymes.

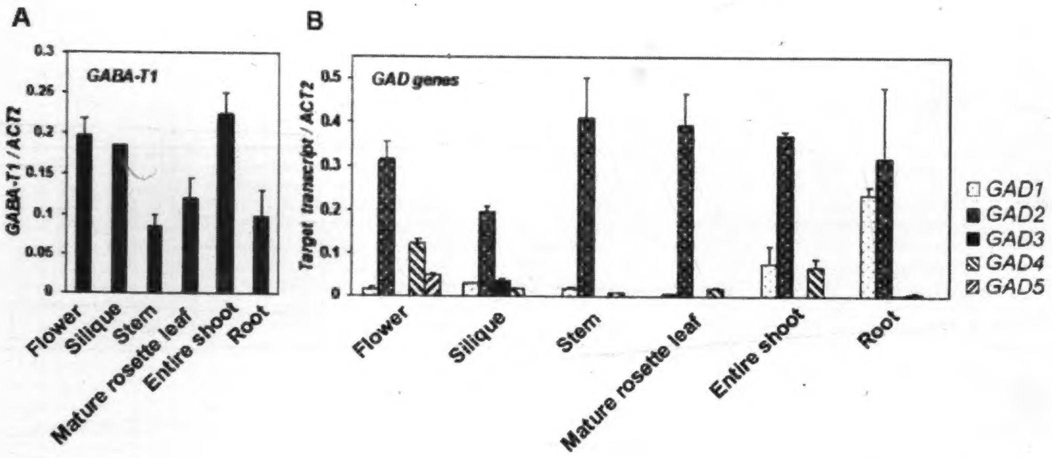


Figure 3.2. The expression of *GABA-T1* and 5 homologous *GAD* genes in various organs in *Arabidopsis*.

The organs used for RNA extractions were harvested from 40-day-old plants grown on soil under the standard growth condition. The samples for entire shoot and root were harvested from 17-day-old plants grown semihydroponically on liquid 0.5X MS media. The abundance of each transcript was measured by quantitative real time RT-PCR and normalized against *ACT2* (*Actin2*). (A) Expression profile of *GABA-T1*. (B) Expression profile of 5 homologous *GAD* genes. Results shown are the means ± S.D. of two biological replicates.

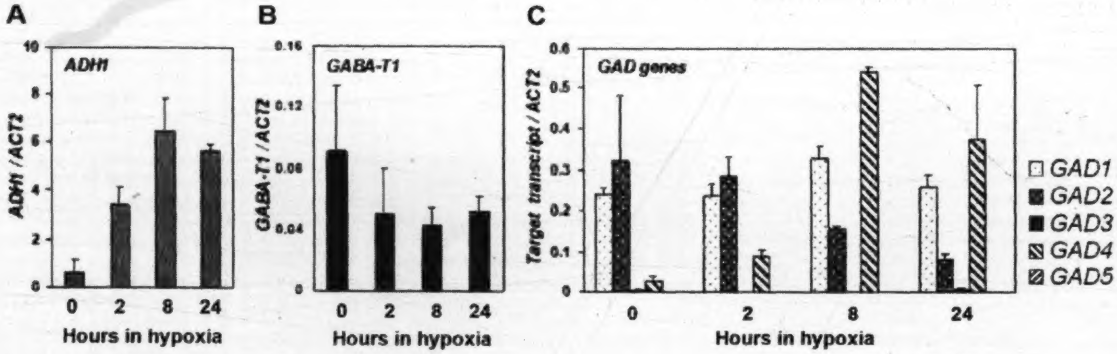


Figure 3.3. Transcriptional modulation of *GABA-T1* and 5 homologous *GAD* genes in response to hypoxic conditions.

Root tissues of the 17-day-old plants treated for hypoxic conditions (5% O_2) for the indicated period were used for RNA extractions. The expression of alcohol dehydrogenase gene (*ADH1*) was analyzed along with *GABA-T1* and 5 homologous *GAD* genes. The abundance of each transcript was measured by quantitative real time RT-PCR and normalized against *ACT2* (*Actin2*). (A) Changes in the transcript abundance of *ADH1*. (B) Changes in the transcript abundance of *GABA-T1*. (C) Changes in the transcript abundance of 5 homologous *GAD* genes. Results shown are the means ± S.D. of two biological replicates.

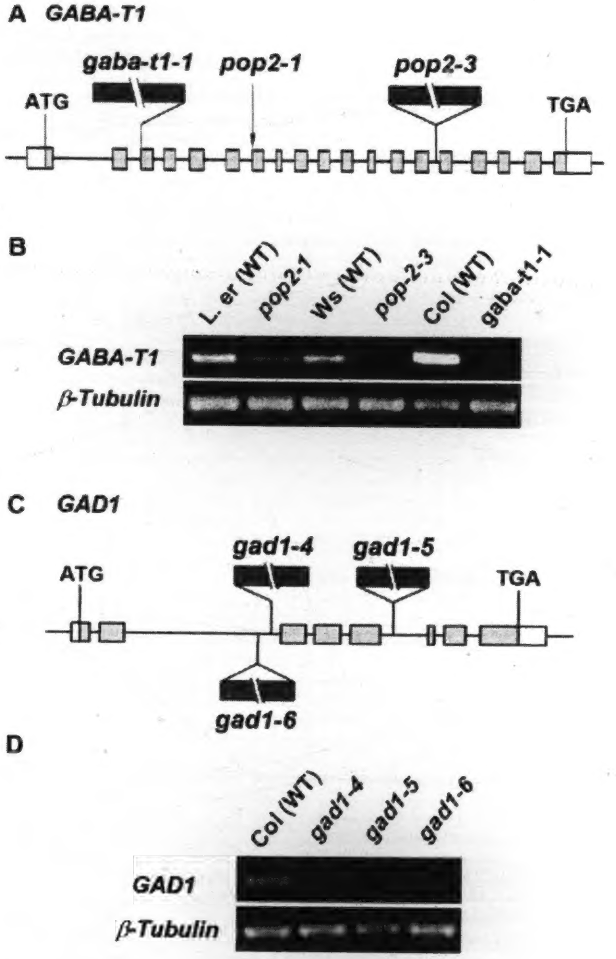

A GABA-T1
gaba-t1-1
pop2-1
pop2-3
ATG
TGA
B
L. er (WT)
pop2-1
Ws (WT)
pop-2-3
Col (WT)
gaba-t1-1
GABA-T1
β-Tubulin
C GAD1
gad1-4
gad1-5
ATG
TGA
gad1-6
D
Col (WT)
gad1-4
gad1-5
gad1-6
GAD1
β-Tubulin

Figure 3.4. Molecular characterization of *gaba-t1* and *gad1* mutants.

(A) The gene structure of *Arabidopsis GABA-T1* and the sites of T-DNA insertions in *gabat1-1* and *pop2-3* and the site of point mutation in *pop2-1*. (B) RT-PCR analysis showing the absence of *GABA-T1* transcript in *gaba-t1-1* and *pop2-3*. (C) The structure of *Arabidopsis GAD1* and the sites of T-DNA insertions in each *gad1* mutant. (D) RT-PCR analysis showing the absence of *GAD1* transcript in each *gad1* mutant. For RT-PCR analysis, total RNA isolated from roots of 17-day-old plants grown semihydroponically on liquid 0.5X MS media was used. PCR was performed with the synthesized first-strand cDNA and with the gene-specific primers flanking the T-DNA insertion junctions.

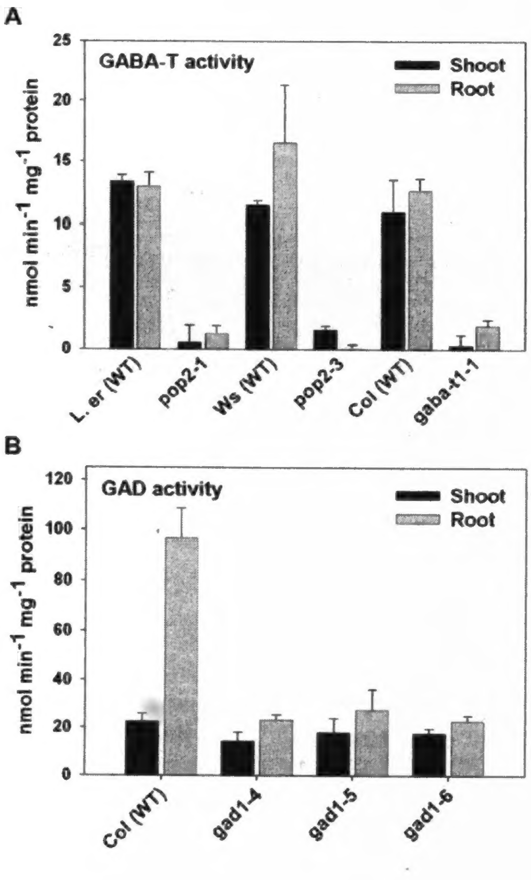


Figure 3.5. Reduced enzyme activity in *gaba-t1* and *gad1* mutants.

The shoot and root tissues of 17-day-old plants grown semihydroponically on liquid 0.5X MS media were harvested and used for protein extractions. (A) Reduction in GABA-T activity in *gaba-t1* mutants. (B) Reduction in GAD activity in roots of *gad1* mutants. The results shown are the means ± S.D. of the triplicate samples.

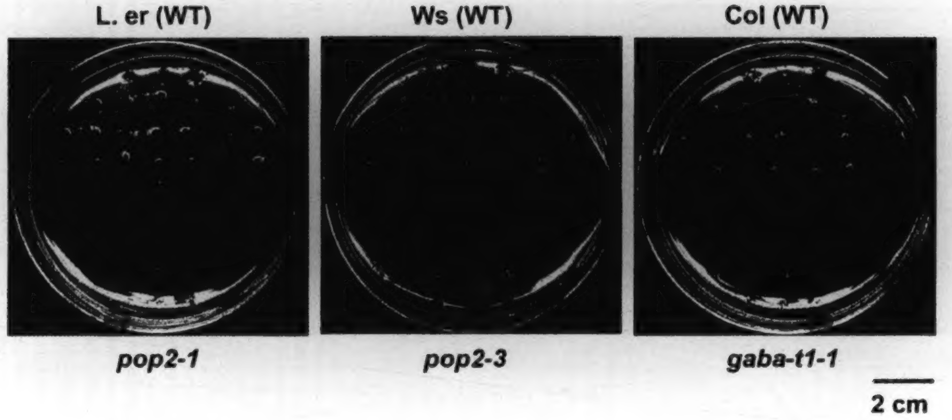
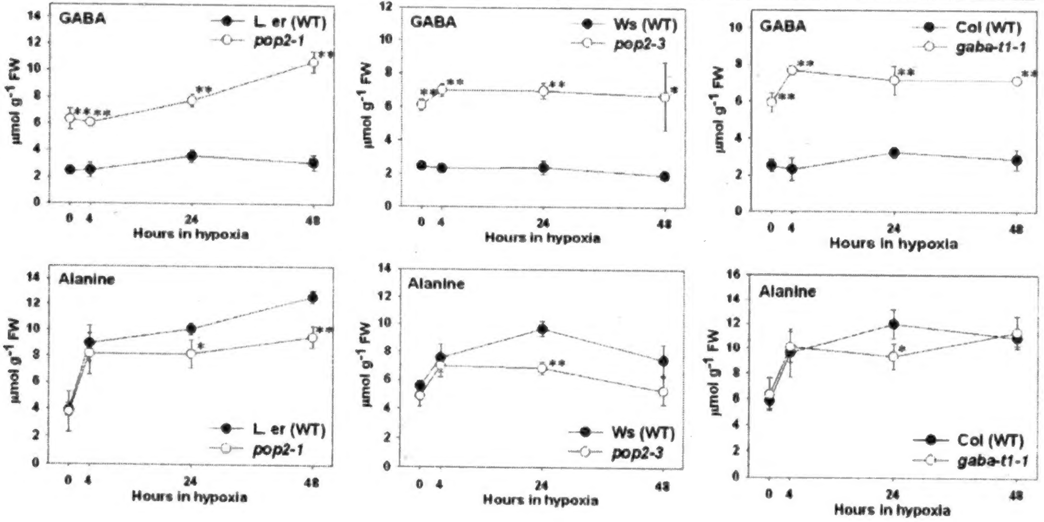


Figure 3.6. Conditional growth defects of *gaba-t1* mutants on nitrogen-free 0.5X MS media with the supplementation of GABA.

Seeds were surface-sterilized and plated on controlled 0.5X MS media with no inorganic nitrogen (NO_3^- and NH_4^+). To these media, GABA was supplemented to a final concentration of 10 mM. *gaba-t1-1* mutants and their corresponding WT lines were grown on these media for 2 weeks under the normal growth condition.

Root



Shoot

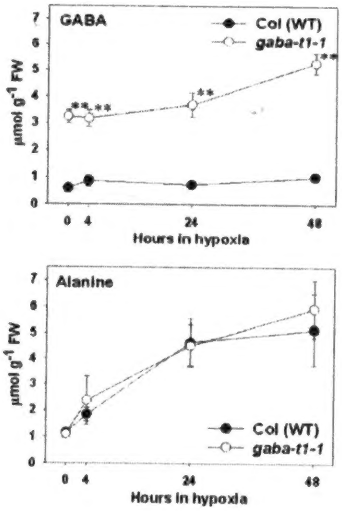


Figure 3.7. Changes in metabolite levels in *gaba-t1* mutants and their corresponding WT lines during the hypoxic treatments.

Metabolites were extracted from the plants treated for hypoxic conditions (5% O_2) for the indicated period. The levels of metabolites were measured by enzyme-linked assays. The results shown are the means ± S.D. of three biological replicates. * and ** indicates $P < 0.05$ and $P < 0.01$ (Student's t-test), respectively, for the comparison of *gaba-t1* mutants with their corresponding WT lines.

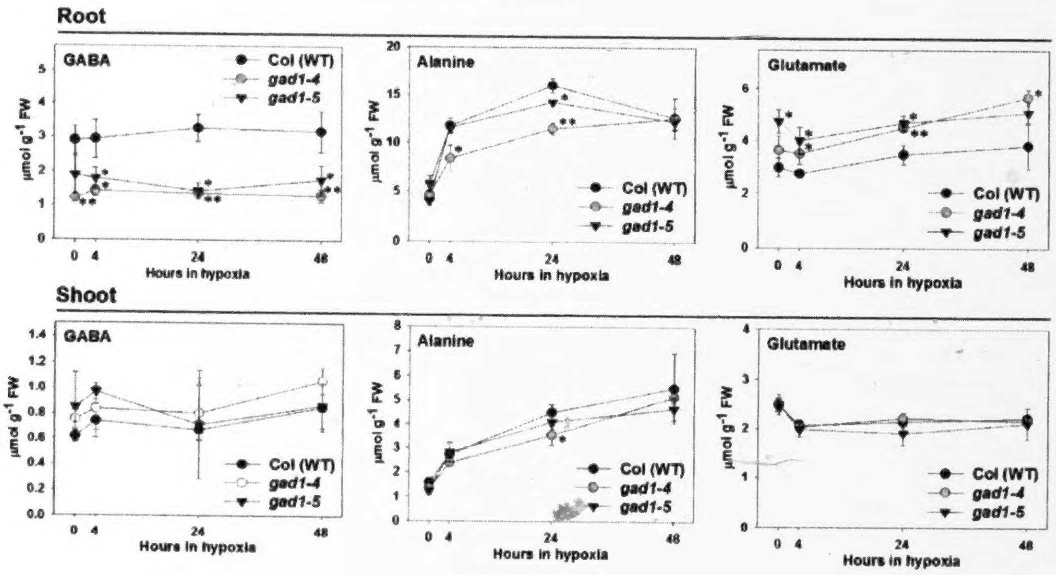


Figure 3.8. Changes in metabolite levels in *gad1* mutants and WT line during the hypoxic treatments.

Metabolites were extracted from the plants treated for hypoxic conditions (5% O_2) for the indicated period. The levels of metabolites were measured by enzyme-linked assays. The results shown are the means ± S.D. of three biological replicates. * and ** indicates $P < 0.05$ and $P < 0.01$ (Student's t-test), respectively, for the comparison of *gad1* mutants with WT line.

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4. NAD(H)-dependent glutamate dehydrogenase is essential for survival of *Arabidopsis thaliana* during dark-induced carbon starvation.

4.1. Introduction

Carbon (C) and nitrogen (N) metabolism is fundamental to plant growth and development. The complex coordination of C and N metabolism is achieved by sensing the cellular C/N balance and regulating the transcription of many genes involved in photosynthesis, respiration and N assimilation (Stitt and Krapp, 1999; Coruzzi and Zhou, 2001; Palenchar *et al.*, 2004; Plaxton and Podestá, 2006). Metabolically, this intricate linkage involves the interconversion between keto-acids and amino acids (Lea and Ireland, 1999). Glutamate dehydrogenase (GDH; EC 1.4.1.2) catalyzes *in vitro* the reductive amination of 2-oxoglutarate to glutamate as well as the oxidative deamination of glutamate to 2-oxoglutarate. Since the conversion of these metabolites constitutes the major link between C and N metabolism in plants, GDH appears to play a pivotal role in plant metabolism.

In terms of the genes encoding GDH in plants, there are at least two *GDH* genes in all plant species studied to date, each encoding distinct polypeptides, α or β subunits of GDH (reviewed by Purnell *et al.*, 2005). The random assembling of the α and β subunits in the hexameric holoenzyme yields seven isoforms that can be visualized on a non-denaturing polyacrylamide gel (Loulakakis and Roubelakis-Angelakis, 1991, 1996). In *Arabidopsis,* two *GDH* genes have been cloned and characterized (Turano *et al.*, 1997). Studies conducted on these two *Arabidopsis GDH* genes suggest that they are responsible for the most of GDH activity in *Arabidopsis* although two other genes were subsequently identified and annotated as putative *GDH* genes (Purnell *et al.*, 2005; Fontaine *et al.*, 2006). Also, expression of the two *Arabidopsis GDH* genes has been shown to be dependent on the organ type, developmental stage and nutritional conditions (Turano *et al.*, 1997). Moreover, the mitochondrial localization of GDH has been demonstrated by subcellular fractionation (Turano *et al.*, 1996, 1997). Immunohistochemical studies showed the specific localization

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of GDH in the mitochondria of phloem companion cells and on occasion in the cytoplasm of these cells (Dubois *et al*., 2003; Tercé-Laforgue *et al*., 2004a; Fontaine *et al*., 2006).

GDH had been originally viewed as the major ammonium assimilatory enzyme; however, Lea and Miflin (1974) demonstrated that glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle was the major route for ammonium assimilation in plants. Since then, the *in vivo* direction of the GDH reaction and hence the role of the enzyme has remained ambiguous despite much research targeted at addressing this question. Currently, it seems accepted that GDH is not essential for the primary N assimilation; however, the potential of GDH playing a role in ammonium assimilation under certain physiological conditions cannot be discarded (Dubois *et al*., 2003). For instance, the up-regulation of *GDH* in response to elevated ammonium levels suggests that GDH is important in the detoxification of ammonium by assimilating some of the excess ammonium ions (Tercé-Laforgue *et al*., 2004a, 2004b). A recent study also reported that ammonium assimilation could be attributed to GDH under salt stress conditions (Skopelitis *et al*., 2006). On the other hand, the catabolic role of GDH has been evident in several labeling experiments using carrot cell suspensions (Robinson *et al*., 1991), isolated potato mitochondria (Aubert *et al*., 2001) and the transgenic tobacco plants possessing higher GDH activity (Purnell and Botella, 2007). These results suggest that GDH functions to funnel the C skeletons of glutamate into the TCA cycle for energy production under C limiting conditions. The strict regulation of *GDH* genes by the cellular C availability further supports this notion (Robinson *et al*., 1991; Melo-Oliveira *et al*., 1996; Masclaux-Daubresse *et al*., 2002; Restivo, 2004).

The analysis of mutant plants deficient for a specific enzyme is often critical in elucidating metabolic pathways and the *in vivo* function of the enzyme. GDH-deficient mutants have been isolated in maize (Magalhaes *et al*., 1990; Pryor, 1990) and in *Arabidopsis* (Melo-Oliveira *et al*., 1996; Fontaine *et al*., 2006). The maize GDH-deficient mutant, which had 10 to 15- fold decreases in root GDH activity, did not allow the determination of the anabolic role of GDH. Although a significant decrease in the rate of $^{15}NH_4^+$ assimilation was observed in the mutant roots, the inhibition of ammonium assimilation by a potent GS inhibitor, methionine sulfoximine (MSX), argued against the N assimilatory role of GDH (Magalhaes *et al*., 1990). In another study, feeding

[^{15}N]glutamate to the root system resulted in reduced [^{15}N]glutamate catabolism in the maize *gdh* mutant, indicating the catabolic role of GDH (Stewart *et al*., 1995). However, these studies lacked an isogenic control line, making the results difficult to interpret. Conversely, the growth retardation of *Arabidopsis GDH1*-deficient mutant (*gdh1-1*) grown on high inorganic N media suggested a non-redundant role of GDH for ammonium assimilation under the conditions of excess inorganic N (Melo-Oliveira *et al*., 1996). Nonetheless, considering the exceptionally high concentration of inorganic N used in their study, the suggested ammonium-assimilating role of GDH seems questionable under physiologically relevant conditions.

It should be noted that all the *gdh* mutants identified to date were not true GDH-null mutants because plants contain multiple *GDH* genes, each encoding either α or β of the GDH subunits. Consequently, the elucidation of the role of GDH has awaited the isolation of a mutant lacking both of the subunits. Here, we report the isolation and characterization of *gdh1* mutant, *gdh2* mutant and the *gdh* double knockout mutant in *Arabidopsis*. The susceptibility of the double knockout mutant to dark-induced C limiting conditions suggests the potential role of GDH in amino acid breakdown to supply the carbon skeletons of amino acids to the respiratory pathway.

4.2. Materials and Methods

4.2.1. Plant materials and plant growth conditions

The Columbia ecotype of *Arabidopsis thaliana* was used in all experiments. The T-DNA mutant lines, Salk_042736 (*gdh1-2*), SAIL_191_F12 (*gdh1-3*), SAIL_560_C10, Salk_102709, Salk_102710, Salk_102711 (*gdh2-1*), SAIL_192_B08, were obtained from Arabidopsis Biological Resource Center (ABRC). The mutant plants homozygous for the T-DNA insertions were isolated from the obtained seed stocks using the PCR screening protocol described at Salk institute web page (http://signal.salk.edu.). Typically, plants were grown on potting mixture, MetroMix 220 (Scotts Co, Marysville, OH, USA), at 22ºC and at approximately 150 μmol m^{-2} sec^{-1} light (with white fluorescent light; measured at the soil surface or petri dish surface levels) on a 16-h light/ 8-h dark cycles unless otherwise indicated. For some experiments, plants were also grown on vertical plates containing sucrose-free 0.5X MS media on the similar light condition as described above. The root tissues used in this study were harvested from 17-day-old plants grown on 0.5X MS media with 0.4 % agar for 10 days and subsequently grown on the liquid 0.5X MS media for 7 days as described (Miyashita *et al*., 2007).

4.2.2. Dark treatment

To test the survival of plants under prolonged dark conditions, plants were initially grown under the normal light/dark cycle as described above and then transferred to darkness for specified days. For this test, plants grown on soil for 25 days and the plants grown on vertical plates containing sucrose-free 0.5X MS media for 14 days were used. To assess the survival of the plants, the viability of shoot apex was checked after a 5-day recovery period.

To study the response of GDH to the prolonged dark conditions, plants grown in the dark for up to 4 days were also used for RNA and protein extractions. For this purpose, 32-day-old soil-grown plants and 18-day-old plate-grown plants were used for the dark treatments. In addition, 18-day-old plate-grown plants of WT as well as *gdh* mutant lines were used for the dark treatment, and the aboveground tissues were harvested and used for metabolite extractions.

4.2.3. Growth experiments on controlled N media

To characterize the growth phenotype of the *gdh* mutants, plants were grown on controlled 0.5X MS media containing 1 % sucrose and 0.6 % agar with varying concentrations of inorganic N (either KNO_3:NH_4NO_3 =1:1 or NH_4Cl only). To assess the ability of the plants to utilise glutamate as a N source, 5 mM glutamate was added to the controlled 0.5X MS media containing the varying amount of inorganic N (KNO_3:NH_4NO_3 =1:1). In addition, the growth of the plants were tested on the controlled 0.5X MS media containing either 5 mM of glutamate, glutamine, aspartate or alanine as the sole N source. In all experiments, triplicates were used, and the fresh weight and the dry weight of 12 plants were measured after a 2-week-growth period.

4.2.4. RT-PCR and quantitative real time RT-PCR analyses

RNA extraction, DNase treatment, and first-strand cDNA synthesis were performed as described (Miyashita *et al.*, 2007). For the RT-PCR to demonstrate the absence of the corresponding transcripts in the *gdh* mutant lines, amplifications were performed with the synthesized cDNA as templates and with the following gene-specific primers; *GDH1*, 5'-ACCGAAGCTTTGCTTAACGA-3' and 5'-CTTTAAGCTCCCCAGCCTCT-3', *GDH2*, 5'-GGGAAATCGATTCAGGGTTT-3' and 5'-GCGACTCGGTTAACTCCAAG-3' and *β-Tubulin* (Kang and Singh, 2000).

For quantitative real time RT-PCR, PCR reaction and the SYBR Green detection were performed on a light cycler ABI Prism 7000 (Applied Biosystems). Each reaction was performed in triplicate, and the specific amplification of the target was checked with a heat dissociation protocol (60 ºC to 95 ºC) after the completion of the PCR reaction. The gene-specific primers for real time RT-PCR were designed to amplify 125-150 bp sequences found in the coding regions near the 3'-end of each gene; *GDH1*, 5'-CAAGGCTTTATGTGGGAGGA-3' and 5'-TGAGCCACACGATTAACACC-3', GDH2, 5'-GGATTCATGTGGGAAGAGGA-3' and 5'-GCGACTCGGTTAACTCCAAG-3', ACT2, 5'-TATCGCTGACCGTATGAGCA-3' and 5'-TTACCTGCTGGAATGTGCTG-3'. The amplified fragments were cloned into pCR2.1-TOPO vector (Invitrogen). To quantify the transcripts of each gene, the copy numbers of each target were determined by the standard curve constructed using the diluted plasmid samples. The determined copy numbers of *GDH1* and *GDH2* were then normalized against the copy numbers of *ACT2* transcripts.

4.2.5. Enzyme assays

Protein extractions and enzyme assays were performed as described (Turano *et al.*, 1996, 1997), however the assay was slightly modified to measure the activity using a 96-well ELISA plate reader, SpectraMax+ (Molecular Devices, Sunnyvale, CA, USA). In this study, the reductive amination was routinely measured as the activity of GDH. The protein concentrations in each protein extract were determined with protein assay kit (Bio-Rad). For staining non-denaturing gels for GDH activity, proteins were separated by non-denaturing polyacrylamide gel electrophoresis using a Mini-Protein II system (Bio-

Rad) as described (Turano *et al.*, 1997). The polyacrylamide gels were then stained for the oxidative deamination activity of GDH as described (Turano *et al.*, 1996).

4.2.6. Metabolite assays

For amino acid extractions, samples were ground to a fine powder in liquid N_2 using a mortar and a pestle. Amino acids were extracted with a methanol chloroform water (12:5:3) (MCW) mixture at 4°C for 1 h. 150 μl water and 100 μl chloroform were then added to 500 μl of the MCW extract. After the phase-separation, the upper aqueous phase was removed and vacuum dried. The dried samples were re-dissolved in water and used for amino acid analysis. Amino acid samples were analyzed by reverse-phase HPLC of their o-phthaldialdehyde derivatives as described (Sedgwick *et al.*, 1991).

For soluble carbohydrates measurements, the metabolites were extracted with 0.6 M perchloric acids as described (Miyashita *et al.*, 2007). Glucose (HK) Assay Kit, Fructose Assay Kit and Sucrose Assay kit (Sigma, St. Louis, MO, USA) were used to measure the levels of each soluble sugar in the extracts.

4.3. Results

4.3.1. *Arabidopsis GDH* genes and their expression.

Two *GDH* genes, *GDH1* (At5g18170) and *GDH2* (At5g07440), have been previously cloned in *Arabidopsis* (Turano *et al.*, 1997). Phylogenetic analysis indicated that *GDH1* and *GDH2* encode β and α subunit of the NAD(H)-dependent GDH, respectively (Purnell *et al.*, 2005). A database search for additional *GDH* genes has identified a putative *GDH* (At3g03910) encoding β subunit-like protein of NAD(H)-dependent GDH and another distinctive gene encoding NADP(H)-dependent GDH (At1g51720) (Purnell *et al.*, 2005). Therefore, *Arabidopsis* has total of four genes annotated as *GDH*, including two previously characterized *GDH* genes (*GDH1* and *GDH2*), and the other two putative *GDH* genes. In the current study, only the two previously known *GDH* genes, *GDH1* and *GDH2*, were investigated in depth since there had been no clear evidence supporting the contribution of the other *GDH* genes (At3g03910 and At1g51720) to the GDH activity in *Arabidopsis*. Also, in this paper, NAD(H)-dependent GDH is abbreviated simply as "GDH" unless otherwise indicated although plants appear to contain both NAD(H)- and NADP(H)-dependent GDH.

The organ-dependent regulation of *GDH* genes has been previously shown in *Arabidopsis* (Turano *et al*., 1997). In this study, the expression of *GDH1* and *GDH2* was analyzed in various organs using quantitative real time RT-PCR. The results indicted that the two *GDH* genes were differently regulated depending on the organ types. Briefly, the transcripts detected in roots of the plants grown semihydroponically were mostly *GDH2*, whereas those detected in rosette leaves of soil-grown mature plants were mostly *GDH1* (Figure 4.1A). In other organs, both *GDH1* and *GDH2* transcripts were detected at relatively similar levels (Figure 4.1A). In terms of GDH enzyme activity levels, the activity in the roots was exceptionally strong, whereas rosette leaves had the lowest activity levels among the organs tested (Figure 4.1B). The banding pattern of the 7 isoenzymes visualized on a non-denaturing gel also coincided well with the relative abundance of *GDH1* and *GDH2* transcripts; for example, the bands for isoenzyme 5-7 (the hexameric form of GDH is rich in the GDH2 protein) and those for isoenzyme 1-3 (the hexameric form of GDH is rich in GDH1 protein) were evident in roots and rosette leaves, respectively (Figure 4.1C).

4.3.2. Isolation and characterization of *Arabidopsis gdh* mutants.

The T-DNA insertion lines (Alonso *et al*., 2003) obtained from ABRC were screened in search of GDH-deficient mutants. Two mutant lines (*gdh1-2* and *gdh1*-3), each containing a T-DNA insertion in *GDH1*, were isolated. One mutant line (*gdh2-1*), containing a T-DNA insertion in *GDH2*, was also recovered. The exact locations of the T-DNA insertions were confirmed by sequencing the PCR products flanking the insertion junctions (Figure 4.2A, 4.2B). Subsequently, *gdh1-2* and *gdh2-1* were crossed, creating a double knockout mutant (*gdh1-2/gdh2*-1) defective in both *GDH* genes. RT-PCR analysis confirmed the absence of the corresponding gene transcripts in each mutant line (Figure 4.2C). In *gdh2-1* and *gdh1-2/gdh2-1*, the reduction in GDH enzyme activity was evident in both shoots and roots of the 17-day-old plants grown semihydroponically on liquid 0.5X MS media (Figure 4.2D). Typically, GDH activity was negligible in the double knockout mutant both in shoots and roots of the 17-day-old plants (Figure 4.2D) as well as in the stems and leaves of soil-grown mature plants (data not shown). In addition, in-gel GDH staining detected only the single band of isoenzyme 1 (homohexamer of GDH1 protein) and that of isoenzyme 7 (homohexamer of GDH2 protein) in *gdh2-1* and in the two *gdh1* mutants, respectively (Figure 4.2E). In *gdh1-2/gdh2-1*, no band was visible on the non-denaturing gel in shoots and roots (Figure 4.2E), indicating that NAD(H)-dependent GDH activity in this line was not detectable. These results clearly demonstrate that the T-DNA insertions in each *GDH* gene eliminated the functional gene products in the corresponding mutant lines.

An attempt to measure NADP(H)-dependent activity in the *gdh* mutant plants was also made. Since NADP(H) can be used as a cofactor by NAD(H)-GDH (Bhadula and Shargool, 1991; Turano *et al.*, 1996), a similar reduction in NADP(H)-GDH activity was observed in the *gdh* mutants (Figure 4.3). Nonetheless, a small but finite NADP(H)-dependent activity was detected in both shoots and roots of *gdh1-2/gdh2-1* plants (Figure 4.3) although the NAD(H)-dependent GDH activity was negligible in this line. Therefore, this small activity remaining in *gdh1-2/gdh2-1* suggests the presence of the NADP(H)-dependent GDH in *Arabidopsis*. In contrast to the mitochondrial NAD(H)-GDH, plant NADP(H)-dependent GDH has been proposed to be localized in plastids, but little is known about plant NADP(H)-dependent GDH as only limited evidences have suggested the presence of this enzyme in higher plants (Lea and Thurman, 1972; Bhadula and Shargool, 1991; Turano *et al.*, 1996, 1997).

4.3.3. Induction of GDH by prolonged dark conditions.

The regulation of *GDH* in response to cellular C status has been demonstrated in several plant species, characterized by the induction of *GDH* under dark conditions (Melo-Oliveira *et al.*, 1996; Turano *et al.*, 1997; Mascaux-Daubresse *et al.*, 2002; Restivo, 2004). In this study, the dark-induction of GDH was investigated at the transcript and protein levels in the aboveground tissues of young seedlings grown on sucrose-free 0.5X MS media and in the rosette leaves of soil-grown plants. For the enzyme activity assays and the non-denaturing gel staining, the *gdh* mutant plants were used along with WT plants to distinguish the induction patterns of the two GDH subunits.

In the aboveground tissues of the plate-grown seedlings, the transcriptional induction was observed for *GDH1* after 1 day of the dark treatment (increased 1.75-fold), while a gradual repression was seen for *GDH2* during the dark treatment (Figure 4.4A). An increase in the enzyme activity was noticeable in WT plants during the first 2 days of the treatment (Figure 4.4B). Similarly, *gdh1-2* plants showed an increase in the enzyme activity after 1 day of the treatment, but followed by a small decline at subsequent time points (Figure 4.4B). In *gdh2-1* plants, the enzyme activity was very low compared to that in WT plants, and the activity change during the dark treatment appeared minor at the all time points (Figure 4.4B). In *gdh1-2/gdh2-1* plants, GDH activity was nearly undetectable throughout this time-course experiment (Figure 4.4B). The band pattern seen on the non-denaturing gel supported the changes in GDH activity in each line during the dark treatments (Figure 4.4C).

In the rosette leaves of soil-grown plants, a dramatic increase in the *GDH2* transcripts was detected in response to the dark treatments (increased 6.1-fold after 1 day of the treatment and 24.3-fold after 4 days of the treatment, see Figure 4.4D). On the other hand, *GDH1* expression did not change significantly during the treatments (Figure 4.4D). At the enzyme activity levels, an increase was observed for all lines until day 2 of the treatment except for the double knockout mutant. However, these increases were followed by a subsequent enzyme activity reduction in the WT and *gdh2-1* plants (Figure 4.4E). The decrease in the activity in these lines may be attributed to the inactivation or degradation of the GDH1 protein under prolonged dark conditions. In *gdh1-2* plants, the enzyme activity in rosette leaves was very low compared to that of WT, but the induction in the enzyme activity during the dark treatment continued throughout the time-course of the experiment (Figure 4.4E). The apparent transcriptional up-regulation of *GDH2* supports the large increase in the enzyme activity found in *gdh1-2* plants. The non-denaturing gel staining further substantiated the increase of GDH2 protein during the dark treatments because the bands of isoenzyme 5-6 (rich in GDH2 protein) appeared in the dark-treated WT plants and the intensity of the band of isoenzyme 7 increased after the dark treatments in *gdh1-2* plants (Figure 4.4F). In *gdh1-2/gdh2-1* plants, GDH activity was negligible at all time points (Figure 4.4E) and no band was detected by non-denaturing gel staining (data not shown).

4.3.4. Conditional phenotypes of the *gdh* mutants.

Under optimal growth conditions, all *gdh* mutant lines grew to maturity and did not display noticeable growth phenotypes at any developmental stages. To determine if there is any conditional phenotype of the *gdh* mutant plants, growth experiments under several different environmental conditions were performed.

To assess the catabolic role of GDH during C starvation, survival of the plants subjected to prolonged dark conditions was monitored. In this experiment, plants were initially grown under long-day conditions and subsequently transferred to darkness for various periods of time. The survival rate of the plants was scored after a 5-day recovery period (Figure 4.5A, 4.6A). When soil-grown plants were tested for survival under prolonged dark conditions, an increased susceptibility of *gdh1-2/gdh2-1* plants to the dark treatment was observed (Figure 4.5B). Typically, necrosis was observed at the tip of the rosette leaves in *gdh1-2/gdh2-1* plants after 3 days of the dark treatment, and *gdh1-2/gdh2-1* plants rarely survived the 6-day dark treatment (Figure 4.5B). The single *gdh* mutants, (*gdh1-2* and *gdh2-1*) behaved much like WT plants and the death of these plants was observed after 12 days of the treatment. The similar conditional phenotype of *gdh1-2/gdh2-1* was also observed when the plants were grown on sucrose-free 0.5X MS media (Figure 4.6B). Approximately 30 % of *gdh1-2/gdh2-1* plants survived the 4-day dark treatment and the survival rate decreased to 0 % after 6 days of the treatment (Figure 4.7A). To demonstrate that dark-induced C starvation was the cause of the conditional phenotype of *gdh1-2/gdh2-1* plants, subsets of plants were transferred to 0.5X MS media containing 1 % sucrose before the onset of the dark treatment. The sucrose supplementation rescued the conditional phenotype of *gdh1-2/gdh2-1*, although *gdh1-2/gdh2-1* plants were still slightly more susceptible to dark treatments than WT plants (Figure 4.6C, 4.7B). This suggested that the death of the dark-treated *gdh1-2/gdh2-1* plants were caused by C starvation. Therefore, this conditional phenotype of *gdh1-2/gdh2-1* supports the hypothesis that GDH plays a catabolic role in supplying C source to the respiratory pathway for energy production under the conditions of C deficit. Moreover, the absence of this conditional phenotype in both of the single *gdh* mutants indicates that both of the homohexameric GDH isoenzymes (isoenzyme 1 and 7) catalyze glutamate breakdown under C deficient conditions. Since it is likely that heterohexameric isoenzymes (isoenzyme 2 – 6) also catalyze the glutamate catabolism, both GDH1 and GDH2 proteins play a redundant role under C starvation.

In order to give insights into the physiological role of GDH in other conditions, several growth experiments using the *gdh* mutants were performed. In one experiment, the concentrations of inorganic N (used at the ratio, NO_3^- : NH_4^+ = 2:1) were varied from 0 mM to 30 mM. This dose-response experiment did not result in any growth phenotypes in the *gdh* mutants (Figure 4.8, Table 4.1). However, the supplementation of 5 mM glutamate as the alternative N source to these media revealed the growth retardation of *gdh2-1* and *gdh1-2/gdh2-1* plants (Figure 4.8, Table 4.1). Because glutamate N has to be utilized to support plant growth under N limiting conditions, the reduced growth of *gdh2-1* and *gdh1-2/gdh2-1* plants can be attributed to the reduced mobilization of glutamate N caused by the diminished GDH activity in these lines. In addition, the growth of the plants were tested on the 0.5X MS media containing 5 mM of either glutamate, glutamine, aspartate or alanine as the sole nitrogen source. On these media of the four amino acids tested, *gdh2-1* and *gdh1-1/gdh2-1* showed reduced growth compared to that of WT (Table 4.2). These results suggest that the mobilization of the amino N of other amino acids were also slowed by the inhibition of GDH. In another experiment, plants were grown on media containing various levels of ammonium as the sole nitrogen source, but the growth of the plants on these media was very poor and no difference was observed between WT and the *gdh* mutant lines (data not shown). Also, the addition of GS inhibitor, MSX (as low as 10 μM), to the growth media resulted in seedling death at the very early stage of development in all lines (data not shown). In a separate experiment, it was confirmed that MSX does not inhibit *in vitro* GDH activity (data not shown). Thus, the results suggest that the involvement of GDH in primary N assimilation is unlikely. Overall, the results obtained through these various experiments suggest that GDH plays a negligible role in ammonium assimilation but support its role in glutamate catabolism.

4.3.5. Metabolic changes under the conditions of dark-induced C starvation.

To monitor the changes in the levels of metabolites under dark conditions, 18-day-old plants grown on sucrose-free 0.5X MS media were dark-treated for various days. The aboveground tissues of the dark-treated plants were then harvested and used for the metabolite analysis. Table 4.3 shows the major metabolic differences found between WT and *gdh1-2/gdh2-1* plants (see Appendix 4.1 for the complete data set). In the untreated plants, the amino acid profiles of WT and *gdh1-2/gdh2-1* plants were similar except for a few amino acids (e.g. ornithine). However, the levels of many amino acids differed substantially between WT and *gdh1-2/gdh2-1* plants under dark conditions. For example, the levels of glutamate increased in WT but decreased in *gdh1-2/gdh2-1* plants during the dark treatment (Table 4.3). Also, alanine and aspartate showed a constant decline in WT, but they accumulated in *gdh1-2/gdh2-1* plants in the dark (Table 4.3). Several other amino acids (i.e. histidine, tyrosine, tryptophan, valine, phenylalanine, isoleucine, leucine and lysine) showed increases under dark conditions, however, the magnitude of the increases were often different between WT and *gdh1-2/gdh2-1* plants; in fact, the levels of tyrosine, valine, isoleucine, leucine and lysine were significantly higher in *gdh1-2/gdh2-1* than in WT plants (Table 4.3). In *gdh1-2* plants, the amino acid profiles were relatively similar to WT plants, whereas the amino acid levels in *gdh2-1* plants were more comparable to *gdh2-1/gdh1-2* plants (Appendix 4.1). Combined with the GDH activity found in the aboveground tissues of the young seedlings (a large reduction in *gdh2-1* but not in *gdh1-2*, see Figure 4.2D, 4.4B), GDH activity appears to be an important factor affecting the levels of many amino acids under prolonged dark conditions. In addition, dark-induced asparagine accumulation occurred in WT and all *gdh* mutant plants. However, the observed asparagine increase in this study was relatively smaller than those reported previously (Lam et al., 2003; Lin and Wu, 2004; Lea et al., 2007), possibly due to the young age of the samples used in this study (18-day-old seedlings grown on sucrose-free 0.5X MS media). For the levels of soluble carbohydrates (i.e. glucose, fructose and sucrose), rapid decreases during the dark treatment were observed in both WT and *gdh1-2/gdh2-1* plants (Table 4.3). This confirmed that the prolonged dark treatment mimicked the C limiting conditions.

4.4. Discussion

In an attempt to elucidate the long-disputed role of plant GDH, the regulation of GDH in *Arabidopsis* was examined and the *Arabidopsis gdh* mutants was characterized, focusing mainly on the role of GDH under dark-induced C deficiency. Although GDH has been extensively studied using biochemical, genetic and physiological approaches, previous attempts did not unequivocally define the *in vivo* role of GDH in plants. The difficulty in elucidating the function of GDH has been in part due to the lack of a true GDH-null mutant. In the current study, *Arabidopsis* mutants defective in *GDH1* and *GDH2* was isolated. The cross between the two mutants allowed us to recover the double knockout mutant whose GDH activity was reduced to negligible levels. In the strict sense, this double knockout mutant was not a true GDH-null mutant due to the potential expression of another putative *GDH* gene (At3g03910) and the presence of NADP(H)-dependent GDH activity. It is possible that this NADP(H)-dependent GDH activity allows the double knockout mutant to grow in the absence of NAD(H)-GDH activity. However, the use of this mutant proved useful to unravel the physiological function of plant GDH.

In order to understand the complex nature of GDH regulation in plants, the levels of *GDH* transcripts, enzyme activity and isoenzyme patterns in several organs of *Arabidopsis* were examined. Our results indicated that the expression of the two *GDH* genes was reciprocal in roots and rosette leaves (Figure 4.1A). This finding supports the previous results that showed high levels of *GDH1* mRNA in leaves but not in roots (Melo-Oliveira *et al.*, 1996). On non-denaturing gels stained for GDH activity, root samples were rich in anodal isoforms (isoenzyme 5-7), whereas cathodal isoforms (isoenzyme 1-3) predominated in leaf samples (Figure 4.1C, Turano *et al.*, 1997; Fontaine *et al.*, 2006). This band pattern difference between roots and leaves can be well explained by the organ-dependent expression of *GDH1* and *GDH2*. The large reduction in GDH activity in the roots of *gdh2-1* plants (Figure 4.2B) and that in the rosette leaves of *gdh1-2* plants (Figure 4.4E) also supports the contrasting regulation of the two *GDH* genes in these tissues. It is now generally accepted that C availability is a key factor controlling the levels of *GDH* transcripts and enzyme activity (Sahulka and Lisá 1980; Robinson *et al.*, 1991; Melo-Oliveira *et al.*, 1996; Athwal *et al.*, 1997; Masclaux-Daubresse *et al.*, 2002; Restivo, 2004). The C starvation triggered by prolonged dark conditions is thus the likely cause of the dark-induction of *GDH* because the up-regulation can be reversed by an externally supplied C source (Melo-Oliveira *et al.*, 1996; Masclaux-Daubresse *et al.*, 2002; Restivo, 2004). In this study, the dark-induction was apparent for

GDH2 in rosette leaves of mature plants (Figure 4.4D). Similarly, about a 20-fold increase in *GDH2* transcripts and a significantly smaller increase in *GDH1* transcripts have been previously reported (Turano *et al*., 1997). On the other hand, the dark-induction of *GDH* genes was distinctly different in the aboveground tissues of young *Arabidopsis* seedlings (Figure 4.4A). This suggests that the developmental stages and/or the tissue types affect the inducibility of *GDH* genes during C starvation. Furthermore, the induction of *Arabidopsis GDH2* has also been found during the senescence process (see Genevestigator Response Viewer, Zimmermann *et al*., 2004). This is consistent with the increase of GDH transcripts and activity in senescing leaves (Masclaux *et al*., 2000; Tercé-Laforgue *et al*., 2004b).

In regard to the physiological role of GDH, two conditional phenotypes of the *gdh* double knockout mutant, both of which are consistent with the catabolic role of GDH, were observed. It has been hypothesized that the glutamate catabolized by GDH enters the TCA cycle as an alternative C source during C starvation (Robinson *et al*., 1991, 1992; Melo-Oliveira *et al*., 1996; Aubert *et al*., 2001; Miflin and Habash, 2002; Dubois *et al*., 2003; Purnell and Botella, 2007). The increased susceptibility of *gdh1-2/gdh2-1* plants to prolonged dark conditions and the phenotype rescue by externally supplied sucrose support this hypothesis. It should be noted that the GDH reaction itself might play a role in energy production as it produces extra NADH, which can be used to produce ATP through oxidative phosphorylation. Under the conditions of C deficit, protein degradation is a key process for recycling the cellular components. In fact, decreases in the soluble protein content were observed during the dark treatment in the crude enzyme extracts (data not shown), and there have been several reports of proteolysis induction during C starvation (Moriyasu and Ohsumi 1996; Brouquisse *et al*., 1998; Thompson and Vierstra, 2005). The amino acids catabolized as alternative energy source during C starvation are likely to derive from the protein turnover through an autophagic process, which is a non-selective bulk degradation of cyctoplasmic components (Klionsky and Emr, 2000; Thompson and Vierstra, 2005). Interestingly, *Arabidopsis* mutants deficient in autophagy exhibit increased susceptibility to C starvation, similar to *gdh1-2/gdh2-1* plants (Hanaoka *et al*., 2002; Thompson *et al*., 2005). The phenotype of these mutants substantiates the importance of proteolysis and the subsequent amino acid degradation for the production of alternate energy source under C limiting conditions. The second conditional phenotype, the reduced utilisation of glutamate N observed in *gdh2-1* and *gdh1-2/gdh2-1* plants, supports the role of GDH in catalyzing the oxidative deamination *in vivo*. The growth of the plants on the media containing glutamate as the sole N source also suggests that the ammonium ions released by the GDH reaction can be readily re-assimilated within the plant. Supporting the breakdown of glutamate by GDH, the transgenic tobacco plants over-expressing the β subunit of GDH exhibited increased $[^{15}N]NH_4^+$ accumulation after $[^{15}N]$glutamate feeding (Purnell and Botella, 2007).

It has been hypothesized that GDH assimilates ammonium to alleviate the toxic effects when the levels of ammonium exceed a certain threshold level (Tercé-Laforgue *et al.*, 2004a, 2004b; Skopelitis *et al.*, 2006). In the current study, the *gdh* mutant and WT plants were shown to be equally susceptible to ammonium when the plants were grown on the media containing various levels of ammonium as the sole nitrogen source (data not shown). Thus, our results did not demonstrate the proposed role of GDH in ammonium detoxification. In ammonium-sensitive species, plant roots uptake ammonium, but most of ammonium ions remain unassimilated and are subsequently excreted from root cells (Britto *et al.*, 2001). This futile transmembrane ammonium cycling can consume substantial amount of cellular energy, lowering the adenylate energy state; therefore, this has been suggested as the cause of ammonium toxicity (Britto *et al.*, 2001). This model may explain the GDH induction by ammonium because GDH appears to be regulated by cellular carbohydrate levels and ultimately by cellular ATP levels (Athwal *et al.*, 1997). However, the role of GDH under toxic levels of ammonium remains unclear. The response of the *gdh* mutant lines to ammonium stress should be studied further to uncover the role of GDH in coping with toxic levels of ammonium.

The observed metabolic changes under the dark-induced C deficiency were somewhat puzzling because both the increase in glutamate levels in WT and the fall of glutamate in *gdh* mutants during the dark treatment can be easily explained by the reductive amination of 2-oxoglutate by GDH (Table 4.3, Appendix 4.1). However, this result is not a definitive proof that glutamate is produced by GDH since glutamate can be metabolized by diverse pathways in plants (Lea and Ireland, 1999; Forde and Lea, 2007).

Considering the results of previous studies and the current study, an explanation for the observed metabolic changes by the catabolic role of GDH is contemplated. The two unexpected results that need to be addressed are the fall in glutamate levels and the significant accumulation of several amino acids in the dark-treated *gdh1-2/gdh2-1* mutants (Table 4.3). The observed increase in the levels of several amino acids in the dark conditions may be due to the result of increased protein degradation under C deficient conditions, and GDH may play a role in the breakdown of amino acids to provide alternate energy sources (Figure 4.9). It should be noted that the conversion of glutamate to 2-oxoglutarate by GDH does not necessarily result in the decline in glutamate levels if 2-oxoglutarate is immediately used as the substrate for transaminations (Figure 4.9). The exaggerated amino acid accumulation (i.e. alanine, aspartate, tyrosine, valine, leucine, isoleucine and ornithine) in the *gdh* mutants during the dark treatment may have occurred due to the reduced breakdown of these amino acids in the mutants. For example, the initial step of the catabolism of branched-chain amino acids (valine, leucine and isoluecine) occurs in mitochondria and is catalyzed by aminotransferases using 2-oxoglutarate as an amino acceptor (Taylor *et al.*, 2004). Assuming that GDH catabolise glutamate to 2-oxoglutarate, the reduced supply of 2-oxoglutarate in the *gdh* mutants may have inhibited the aminotransferase reaction, causing the branched-chain amino acids to accumulate in the *gdh* mutants. Taylor *et al.* (2004) also suggested that the catabolism of these branched-chain amino acids is important in providing the alternate oxidative phosphorylation energy source during C starvation. Similarly, the accumulation of alanine, aspartate, tyrosine and ornithine in the *gdh* mutants could have been caused by the reduced conversion of these amino acids to the corresponding keto-acids since the aminotransferase reactions require a supply of 2-oxoglutarate. Supporting this idea, the other amino acids that did not over-accumulate in the *gdh* mutants appears degradable in pathways not requiring 2-oxoglutarate, with one exception for lysine, whose first step of degradation is catalyzed by lysine-ketoglutarate reductase which requires 2-oxoglutarate (Tang *et al.*, 1997; Zhu *et al.*, 2002). The retarded growth of *gdh2-1* and *gdh1-2/gdh2-1* on media containing alanine and aspartate as the sole N source is also consistent with the idea that these amino acids are deaminated in a coupled reaction of an aminotransferase and GDH (Table 4.3; Figure 4.9). On the other hand, the fall in glutamate in the dark-treated *gdh* mutants may have been caused by the malfunctioning of the GS/GOGAT cycle in the *gdh* mutants under carbon deficiency, since this pathway requires energy input (ATP and NADH) and a constant supply of 2-oxoglutarate. Indeed, the GS/GOGAT cycle seems more severely affected in the *gdh1-2/gdh2-1* than in WT plants, evidenced by the markedly low levels of glutamine found in *gdh1-2/gdh2-1* plants after 4 days of the dark treatment (Table 4.3). Because the GS/GOGAT cycle is the major pathway of glutamate synthesis under normal conditions (Lea and Ireland, 1999; Miflin and Habash, 2002), the decline in glutamate levels in the *gdh* mutants may have been caused by the inhibition of GS/GOGAT cycle during the dark treatment. Interestingly, the pattern of these metabolic changes observed under dark conditions was quite similar to the metabolic changes observed when GS was inhibited by MSX; for example, glutamine, glutamate, asparagine, aspartate, alanine and serine levels declined, whereas valine, leucine, isoleucine, proline, and threonine levels accumulated most likely due to the enhanced protein degradation (Rhodes *et al.*, 1986). Considering these possibilities, the overall metabolic phenotype of the *gdh* mutants seems better explained by the catabolic role of GDH. Overall, the obtained data suggest that GDH is a key enzyme in the breakdown of not only glutamate but also several other amino acids under the conditions of C deficiency (Figure 4.9), and the proposed role of GDH is consistent with the increased susceptibility of *gdh1-2/gdh2-1* to prolonged dark conditions since the loss of GDH activity could result in the inhibition of the broad amino acid catabolism.

The proposed role of GDH in amino acid breakdown appears important for survival of essentially all cells/tissues under C limiting conditions. However, plants often sacrifice some cells/tissues (in case, whole organs) to allow more important metabolically active cells/tissues to survive severe stress conditions. Considering this, the specific localization of GDH in the phloem companion cells (Dubois *et al.*, 2003; Tercé-Laforgue *et al.*, 2004a; Fontaine *et al.*, 2006) could be a strategy of plants to allow phloem cells to survive and remain active under C deficient conditions because heterotrophic meristems are dependent on the transport of respiration substrates through the phloem. Supporting this idea, more severe damage often occurred to rosette leaves after the dark treatments compared to stems or shoot apical meristems. The mitochondrial localization of GDH also correlates well with the proposed role of the enzyme in funneling the carbon skeletons of amino acids into TCA cycle during C deficiency. Interestingly, Takenaka *et al*. (2007) reported that the ability of GDH proteins to bind to RNA inhibits *in vitro* RNA editing. They reported that this inhibition was relieved by addition of NADH, NADPH and ATP, demonstrating the ability of GDH to bind to these molecules (Takenaka *et al*., 2007). It would be interesting to see if the binding of GDH to NADH and/or ATP regulate *in vivo* GDH activity in mitochondria, reflecting the coordination of GDH activity in accordance with the cellular energy state, similar to the regulation of GDH in animals (Fang *et al*., 2002).

Table 4.1. Growth of plants on 0.5X MS media with the varying concentrations of inorganic N and the effect of glutamate supplementation.

Seeds were sterilized and plated on 0.5X MS media containing the specified concentrations of inorganic N, with or without 5 mM glutamate supplementation. Plants were grown for 2 weeks under the normal growth conditions. 12 plants per line were harvested from one plate, and the fresh weight (FW) was measured. The values are in mg FW/12 plants. The results shown are the means ± S.D. of triplicate plates. ** indicates $P < 0.01$ (Student's t-test) for comparison of *gdh* mutants with WT. The experiment was repeated with similar results.

	5 mM Glutamate supplementation	0 mM N	0.3 mM N	3 mM N	30 mM N
WT	-	2.3 ± 0.6	11.0 ± 1.0	91.7 ± 10.4	200.7 ± 39.3
gdh1-2	-	2.3 ± 0.6	11.7 ± 2.1	98.3 ± 7.0	195.7 ± 31.9
gdh2-1	-	2.7 ± 0.6	11.0 ± 1.0	98.3 ± 7.2	181.3 ± 16.0
gdh1-2 / gdh2-1	-	2.3 ± 0.6	10.7 ± 1.2	91.0 ± 7.0	185.3 ± 25.0
WT	+	36.0 ± 1.0	48.0 ± 1.0	129.7 ± 10.2	185.7 ± 9.8
gdh1-2	+	36.3 ± 1.2	48.0 ± 1.0	136.3 ± 6.7	197.7 ± 27.5
gdh2-1	+	23.0 ± 1.0 **	34.3 ± 1.5 **	114.0 ± 9.2	209.0 ± 33.1
gdh1-2 / gdh2-1	+	19.7 ± 0.6 **	33.0 ± 1.0 **	110.7 ± 6.7	189.0 ± 4.4

Table 4.2. Growth of plants on 0.5X MS media containing amino acids as the sole nitrogen source.

Seeds were sterilized and plated on 0.5X MS media containing 5 mM of the specified amino acids. Plants were grown for 2 weeks under the normal growth conditions. 12 plants per line were harvested from one plate, and the fresh weight (FW) was measured. The values are in mg FW/12 plants. The results shown are the means ± S.D. of triplicate plates. * and ** indicates $P < 0.05$ and $P < 0.01$ (Student's t-test), respectively, for comparison of *gdh* mutants with WT.

	WT	*gdh1-2*	*gdh2-1*	*gdh1-2/gdh2-1*
Glu	61.7 ± 5.0	49.3 ± 3.2	28.3 ± 1.5 **	26.0 ± 1.0 **
Gln	56.0 ± 6.0	43.7 ± 6.7	28.0 ± 1.7 *	28.7 ± 1.5 *
Asp	62.0 ± 6.2	53.7 ± 9.3	33.7 ± 3.8 **	34.7 ± 7.6**
Ala	81.0 ± 10.1	70.0 ± 4.4	60.3 ± 4.9 *	58.0 ± 4.4 *

Table 4.3. Changes in the levels of metabolites under dark conditions.

18-day-old plants grown on sucrose-free 0.5X MS media were used for the dark treatment, and the aboveground tissues of the dark-treated plants were harvested and used for metabolite extractions. The levels of amino acids were measured by HPLC analysis. The levels of soluble carbohydrates were measured by enzyme-linked assays. The values are in µmol g^{-1} FW. The results shown are the means ± S.D. of three biological replicates. n.d.; not detected. * and ** indicates $P < 0.05$ and $P < 0.01$ (Student's t-test), respectively, for comparison of *gdh1-2/gdh2-1* mutant with WT.

	WT				***gdh1-2/gdh2-1***			
Days in dark	**0 day**	**1 day**	**2 day**	**4 day**	**0 day**	**1 day**	**2 day**	**4 day**
Amino acids								
Asp	0.929 ± 0.122	0.538 ± 0.048	0.428 ± 0.022	0.451 ± 0.028	0.775 ± 0.042	1.000 ± 0.041 **	1.352 ± 0.111 **	2.539 ± 0.382 **
Glu	1.984 ± 0.137	2.568 ± 0.169	2.997 ± 0.170	3.029 ± 0.076	2.292 ± 0.320	1.629 ± 0.110 **	1.142 ± 0.108 **	0.640 ± 0.076 **
Asn	3.343 ± 0.408	6.597 ± 0.227	6.884 ± 1.750	5.624 ± 2.545	3.931 ± 0.223	6.827 ± 0.159	7.520 ± 0.262	6.306 ± 1.423
Ser	2.543 ± 0.215	1.549 ± 0.129	1.642 ± 0.031	1.493 ± 0.145	2.497 ± 0.186	2.125 ±0.082 **	1.974 ± 0.147 *	2.349 ± 0.145 **
Gln	6.702 ± 0.820	7.507 ± 0.889	6.962 ± 0.513	3.878 ± 0.669	7.171 ± 0.529	8.069 ± 0.661	5.838 ± 0.495	1.036 ± 0.223 **
His	n.d.	0.214 ± 0.021	0.323 ± 0.021	0.535 ± 0.041	n.d.	0.184 ± 0.016	0.175 ± 0.007 **	0.362 ± 0.052 *
Arg	2.846 ± 0.430	4.421 ± 0.381	5.072 ± 0.281	4.43 ± 0.508	3.764 ± 0.234 *	4.386 ± 0.560	4.284 ± 0.217 *	3.649 ± 0.634
Ala	0.561 ± 0.032	0.464 ± 0.028	0.192 ± 0.027	0.202 ± 0.020	0.663 ± 0.081	1.348 ± 0.156 **	1.066 ± 0.066 **	1.477 ± 0.090 **
Tyr	0.078 ± 0.008	0.378 ± 0.024	0.389 ± 0.027	0.350 ± 0.013	0.101 ± 0.010 *	0.611 ± 0.041 **	1.229 ± 0.136 **	3.270 ± 0.466 **
Trp	0.006 ± 0.009	0.083 ± 0.007	0.227 ± 0.013	0.490 ± 0.018	0.000 ± 0.001	0.065 ± 0.004 *	0.143 ± 0.010 **	0.338 ± 0.060 *
Val	0.428 ± 0.022	1.011 ± 0.073	1.368 ± 0.065	1.431 ± 0.043	0.505 ± 0.023 *	1.090 ± 0.033	1.480 ± 0.104	2.044 ± 0.247 *
Phe	0.032 ± 0.010	0.331 ± 0.017	0.791 ± 0.048	1.479 ± 0.045	0.026 ± 0.002	0.282 ± 0.019 *	0.550 ± 0.032 **	1.087 ± 0.142 *
Ile	0.016 ± 0.010	0.305 ± 0.014	0.459 ± 0.046	0.507 ± 0.035	0.004 ± 0.002	0.349 ± 0.027	0.618 ± 0.049 *	0.989 ± 0.090 **
Leu	0.026 ± 0.012	0.291 ± 0.030	0.334 ± 0.039	0.395 ± 0.037	0.013 ± 0.003	0.423 ± 0.031 **	0.690 ± 0.078 **	0.923 ± 0.094 **
Lys	0.156 ± 0.015	0.606 ± 0.038	0.813 ± 0.050	0.555 ± 0.020	0.161 ± 0.006	0.593 ± 0.040	0.944 ± 0.058 *	1.380 ± 0.106 **
Ornithine	0.160 ± 0.025	0.310 ± 0.034	0.306 ± 0.038	0.178 ± 0.034	0.294 ± 0.010 **	0.777 ± 0.071 **	0.723 ± 0.075 **	0.434 ± 0.019 **
Carbohydrates								
Glucose	2.477 ± 0.230	0.183 ± 0.120	0.108 ±0.106	0.075 ± 0.204	2.862 ± 0.787	0.249 ± 0.155	0.047 ± 0.041	0.131 ± 0.053
Fructose	2.981 ± 0.279	0.540 ± 0.137	n.d.	0.014 ± 0.025	2.839 ± 0.945	0.355 ± 0.284	0.085 ± 0.148	n.d.
Sucrose	0.738 ± 0.279	0.198 ± 0.101	0.205 ± 0.114	0.092 ± 0.072	0.796 ± 0.119	0.154 ± 0.138	0.133 ± 0.018	0.010 ± 0.018

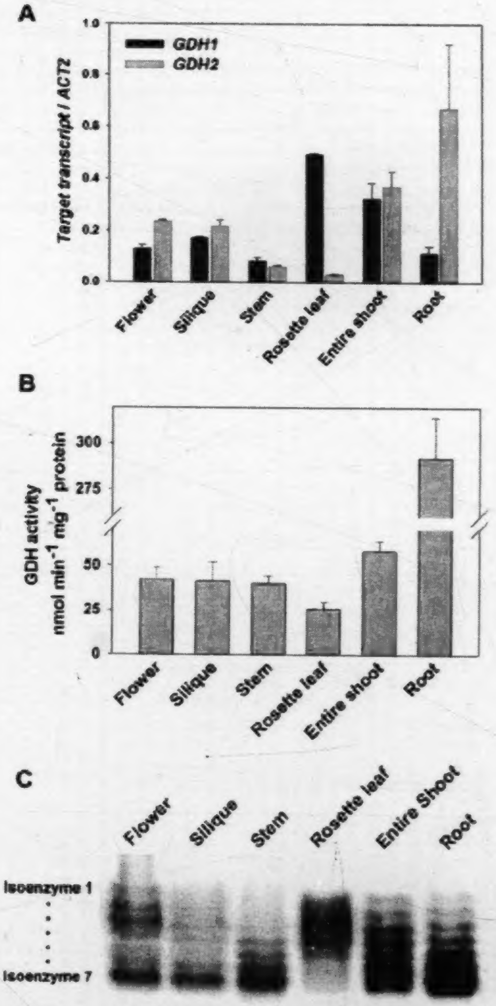


Figure 4.1. Expression of the two *Arabidopsis GDH* genes in various organs.

The organs used for RNA and enzyme extractions were harvested from 40-day-old plants grown on soil under normal growth conditions. The samples for entire shoot and root were harvested from 17-day-old plants grown on liquid 0.5X MS media. (A) Transcriptional profile of *GDH1* and *GDH2* in various organs. The abundance of each transcript was measured by quantitative real time RT-PCR and normalized against *ACT2*. The results shown are the means ± S.D. of two biological replicates. (B) GDH activity found in various organs. The GDH activity was measured for the reductive amination. The results shown are the mean ± S.D. of triplicate samples. (C) Non-denaturing gel stained for GDH activity using the crude extracts from various organs. Crude enzyme extracts were loaded onto a non-denaturing polyacrylamide gel, and proteins were separated by electrophoresis. The gel was subsequently stained for GDH activity. The seven isoenzymes result from the random assembling of α and β subunits encoded by *GDH2* and *GDH1*, respectively.

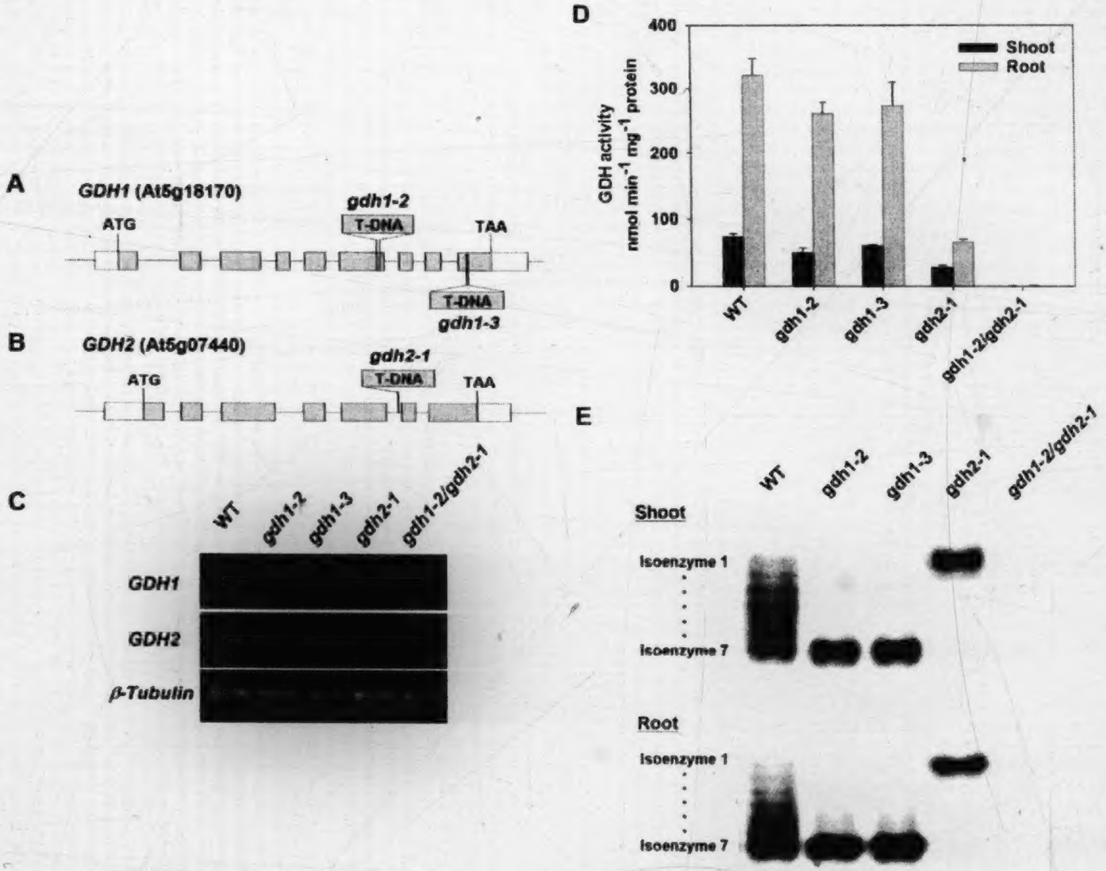


Figure 4.2. Molecular characterization of the *gdh* mutant lines.

(A, B) The gene structure of the two *Arabidopsis GDH* genes and the site of T-DNA insertions in each knockout mutant. Exon regions are shown by shaded boxes. (C) RT-PCR analysis showing the absence of the corresponding transcripts in each *gdh* mutant line. Total RNA was isolated from shoots of the 17-day-old plants grown semihydroponically on liquid 0.5X MS media. PCR was performed with the synthesized first-strand cDNA and with the gene-specific primers flanking the T-DNA insertion junctions. (D) Reduced GDH activity in the *gdh* mutant lines.
The shoot and root tissues of the 17-day-old plants grown semihydroponically on liquid 0.5X MS were harvested and used for protein extractions. The GDH activity was measured for the reductive amination. The results shown are the mean ± S.D. of triplicate samples. (E) Non-denaturing gel stained for GDH activity indicating the absence of the corresponding gene products in each *gdh* mutant line. Crude enzyme extracts were loaded onto a non-denaturing polyacrylamide gel, and proteins were separated by electrophoresis. The gel was subsequently stained for GDH activity.

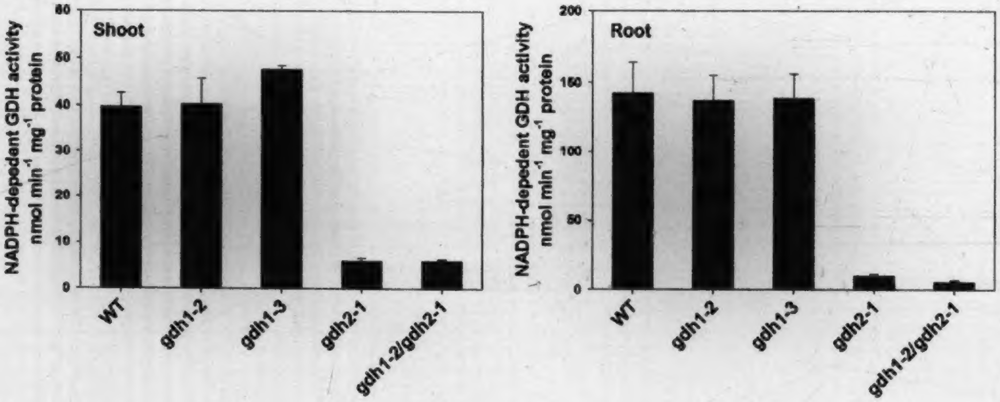
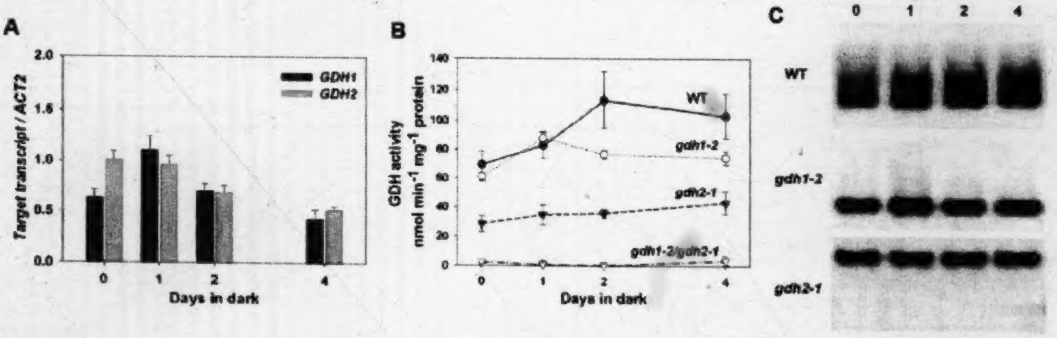


Figure 4.3. NADPH-dependent GDH activity in *gdh* mutants.

The shoot and root tissues of the 17-day-old plants grown semihydroponically on liquid 0.5X MS were harvested and used for protein extractions. The NADPH-dependent GDH activity was measured for the reductive amination. The results shown are the mean ± S.D. of triplicate samples.

Aboveground tissues of young seedlings



Rosette leaves

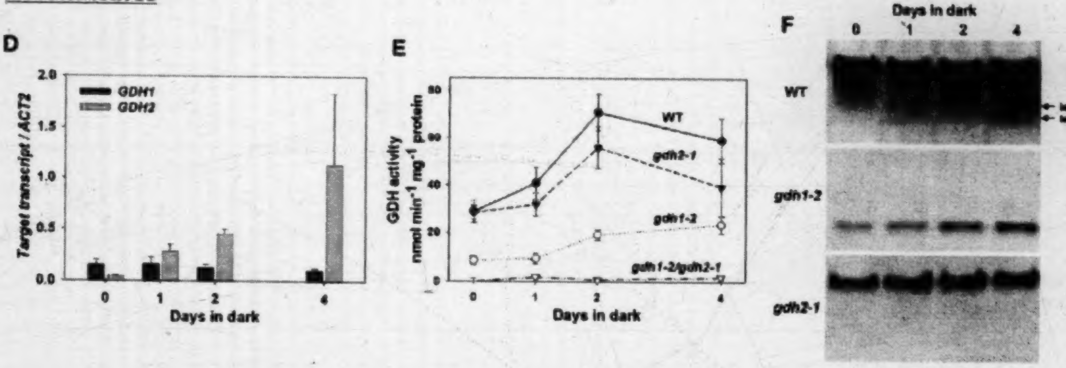


Figure 4.4. Modulation of GDH expression in response to dark conditions.

Plants grown on sucrose-free 0.5X MS media for 18 days were used for the dark treatment. The aboveground tissues of the dark-treated plants were harvested and used for RNA and protein extractions. 32-day-old soil-grown plants were also subjected to dark conditions, and the rosette leaves were harvested and used for RNA and protein extractions. (A, D) Changes in the transcript levels under dark conditions. The levels of each transcript were measured by quantitative real time RT-PCR and normalized against *ACT2*. (B, E) Changes in GDH enzyme activity under dark conditions. All results shown are the means ± S.D. of three biological replicates. (C, F) Non-denaturing gel stained for GDH activity using crude extracts from dark-treated plants. Crude enzyme extracts were loaded onto a non-denaturing polyacrylamide gel, and proteins were separated by electrophoresis. The gel was subsequently stained for GDH activity.

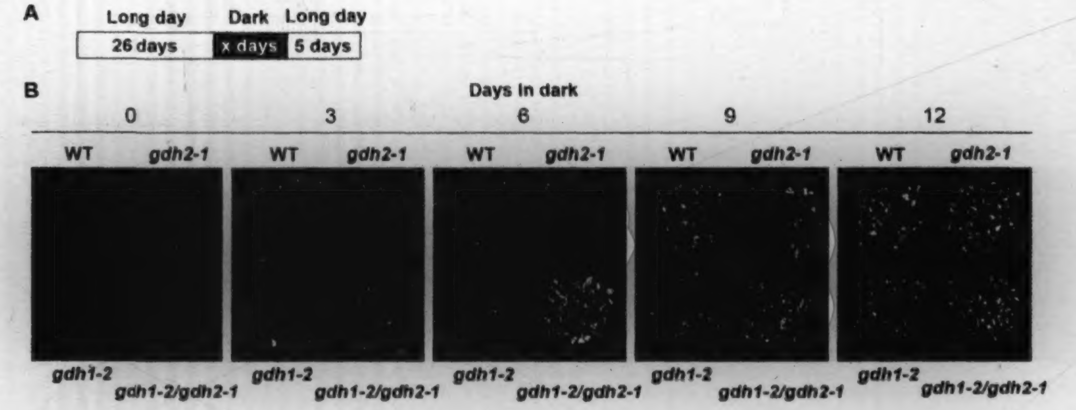

A
Long day
Dark
Long day
26 days
x days
5 days
B
Days in dark
0
3
6
9
12
WT
gdh2-1
WT
gdh2-1
WT
gdh2-1
WT
gdh2-1
WT
gdh2-1
gdh1-2
gdh1-2/gdh2-1
gdh1-2
gdh1-2/gdh2-1
gdh1-2
gdh1-2/gdh2-1
gdh1-2
gdh1-2/gdh2-1
gdh1-2
gdh1-2/gdh2-1

Figure 4.5. Increased susceptibility of *gdh1-2/gdh2-1* plants to prolonged dark treatments.

(A) Schematic representation of the experiment testing the survival of plants under prolonged dark conditions. Plants were grown and treated as indicated and the survival was scored after a 5-day recovery period. (B) Photographs showing the susceptibility of *gdh1-2/gdh2-1* plants to dark conditions. 9 plants were grown in each pot. The experiment was repeated with similar results.

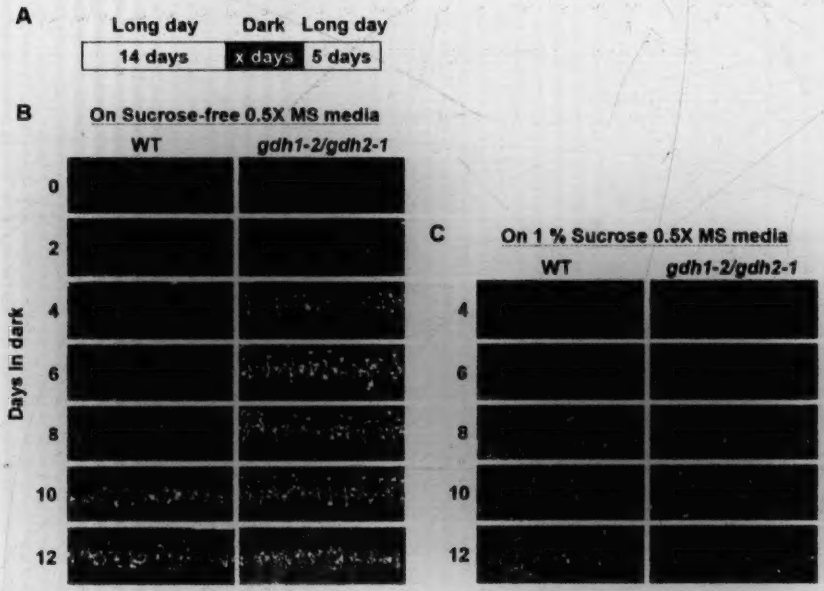

A
Long day
Dark
Long day
14 days
x days
5 days
B
On Sucrose-free 0.5X MS media
WT
gdh1-2/gdh2-1
Days in dark
0
2
4
6
8
10
12
C
On 1 % Sucrose 0.5X MS media
WT
gdh1-2/gdh2-1
4
6
8
10
12

Figure 4.6. Phenotype rescue of *gdh1-2/gdh2-1* by externally supplied sucrose.

(A) Schematic representation of the experiment testing the survival of plants under prolonged dark conditions. (B) Photographs showing the susceptibility of *gdh1-2/gdh2-1* plants to dark conditions. The plants grown on sucrose-free 0.5X MS media were subjected to the dark treatments on the same sucrose-free 0.5X MS media. (C) Phenotype rescue by externally supplied sucrose. The plants grown on sucrose-free 0.5X MS media were transferred to 1 % sucrose 0.5X MS media prior to the dark treatment. All experiments were performed in triplicate and repeated with similar results.

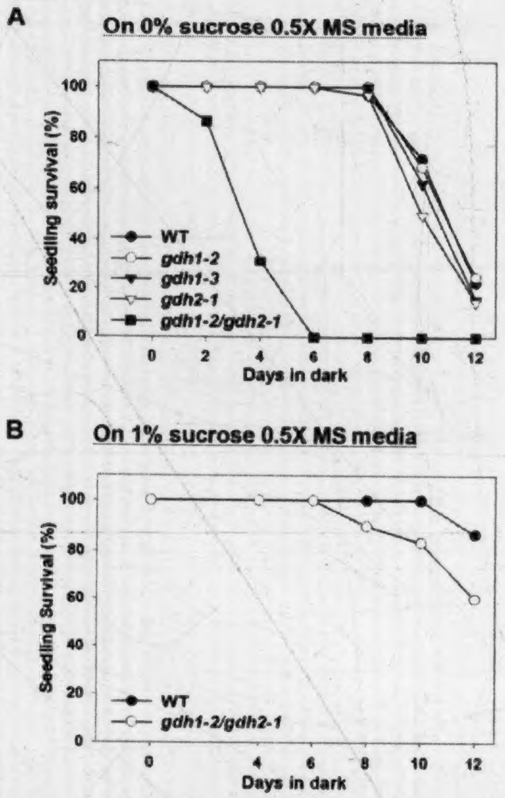


Figure 4.7. Survival rates of plants subjected to prolonged dark conditions.

Plants were grown on sucrose-free 0.5X MS media for 14 days and used for the dark treatments. Survival rates were calculated from the total of the triplicate plates each containing 18 plants. (A) The survival rates of plants subjected to prolonged dark conditions on the sucrose-free 0.5X MS media. (B) The survival rates of plants subjected to prolonged dark conditions on 1 % sucrose 0.5X MS media. Plants were transferred to the 1 % sucrose 0.5X MS media prior to the onset of the dark treatment.

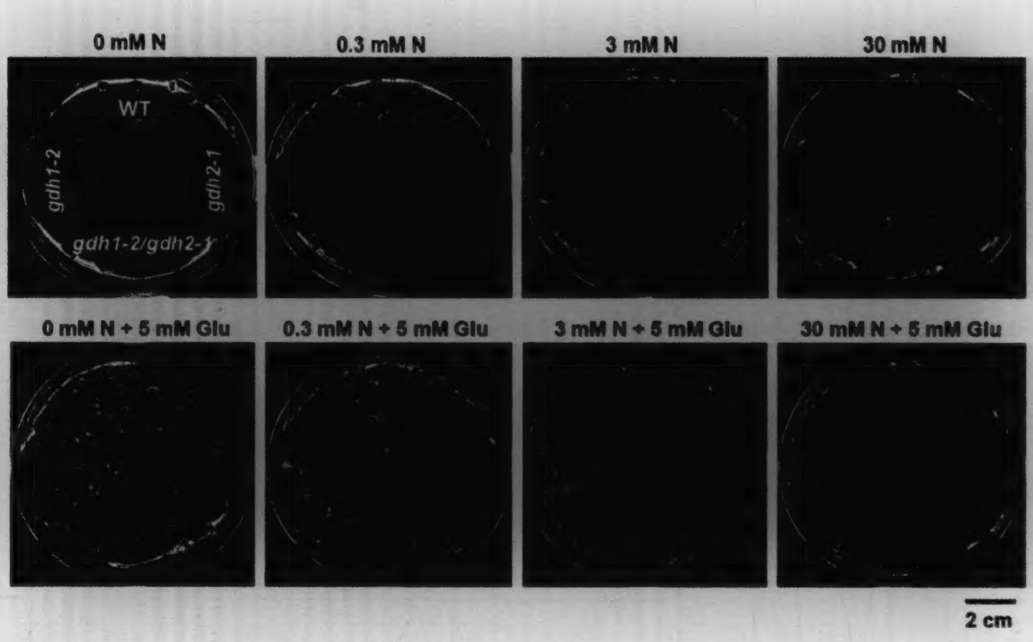

0 mM N
0.3 mM N
3 mM N
30 mM N
0 mM N + 5 mM Glu
0.3 mM N + 5 mM Glu
3 mM N + 5 mM Glu
30 mM N + 5 mM Glu

Figure 4.8. Growth of the *gdh* mutant lines on 0.5X MS media with the varying concentrations of inorganic N in the presence or absence of glutamate supplementation.

Seeds were sterilized and plated on the 0.5X MS media containing the specified concentrations of inorganic N, with or without 5 mM glutamate supplementation. Plants were grown for 2 weeks under the normal growth conditions and photographed.

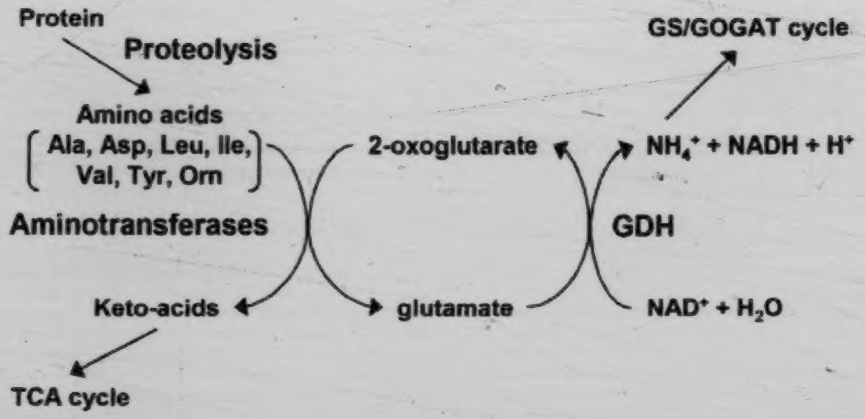

Protein
Proteolysis
Amino acids
Ala, Asp, Leu, Ile, Val, Tyr, Orn
Aminotransferases
Keto-acids
TCA cycle
2-oxoglutarate
glutamate
GDH
GS/GOGAT cycle
NH4+ + NADH + H+
NAD+ + H2O

Figure 4.9. Proposed role of GDH in amino acid breakdown.

The aminotransferase reactions for the conversion of the indicated amino acids to their corresponding keto-acids were confirmed by the AraCyc pathways (http://www.arabidopsis.org/biocyc/index.jsp). Orn indicates ornithine. GS/GOGAT cycle; glutamine synthetase/glutamate synthase cycle.

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Appendix 4.1. Changes in the levels of amino acids under dark conditions.
18-day-old plants grown on sucrose-free 0.5X MS media were used for the dark treatment, and the aboveground tissues of the dark-treated plants were harvested and used for metabolite extractions. The levels of amino acids were measured by HPLC analysis. The values are in µmol/g FW. The results shown are the means ± S.D. of three biological replicates. n.d.; not detected. * and ** indicates $P < 0.05$ and $P < 0.01$ (Student's t-test), respectively, for comparison of gdh mutants with WT.

	WT				*gdh1-2/gdh2-1*				*gdh1-2*				*gdh2-1*			
	0 day	1 day	2 day	4 day	0 day	1 day	2 day	4 day	0 day	1 day	2 day	4 day	0 day	1 day	2 day	4 day
Aspartate	0.929 ± 0.122	0.538 ± 0.048	0.428 ± 0.022	0.451 ± 0.028	0.775 ± 0.042	1.000 ± 0.041 **	1.352 ± 0.111 **	2.539 ± 0.382 **	0.816 ± 0.076	0.531 ± 0.039	0.398 ± 0.006	0.413 ± 0.009	0.839 ± 0.104	0.950 ± 0.038 **	1.120 ± 0.049 **	2.204 ± 0.086 **
Glutamate	1.984 ± 0.137	2.568 ± 0.169	2.997 ± 0.170	3.029 ± 0.076	2.292 ± 0.320	1.629 ± 0.110 **	1.142 ± 0.108 **	0.640 ± 0.076 **	1.737 ± 0.128	2.600 ± 0.149	2.648 ± 0.036 *	3.039 ± 0.154	1.782 ± 0.129	1.651 ± 0.053 **	1.765 ± 0.058 **	2.130 ± 0.141 **
Asparagine	3.343 ± 0.408	6.597 ± 0.227	6.884 ± 1.750	5.624 ± 2.545	3.931 ± 0.223	6.827 ± 0.159	7.520 ± 0.262	6.306 ± 1.423	4.984 ± 0.296 **	6.867 ± 0.227	6.376 ± 1.460	5.860 ± 1.856	4.416 ± 0.136 *	8.904 ± 0.044	7.546 ± 0.161	5.617 ± 2.310
Serine	2.543 ± 0.215	1.549 ± 0.129	1.642 ± 0.031	1.493 ± 0.145	2.497 ± 0.188	2.125 ±0.082 **	1.974 ± 0.147 *	2.349 ± 0.145 **	2.586 ± 0.242	1.528 ± 0.116	1.258 ± 0.054 **	1.237 ± 0.082 *	2.419 ± 0.319	2.006 ± 0.158 *	1.935 ± 0.095 **	2.798 ± 0.242 **
Glutamine	6.702 ± 0.820	7.507 ± 0.889	6.982 ± 0.513	3.878 ± 0.669	7.171 ± 0.529	[illegible]	5.838 ± 0.495	1.036 ± 0.223 **	8.342 ± 0.332 *	8.284 ± 0.652	5.299 ± 0.336 **	3.084 ± 0.517	7.067 ± 0.175	8.294 ± 0.398	7.287 ± 451	3.002 ± 0.364
Histidine	n.d.	0.214 ± 0.021	0.323 ± 0.021	0.535 ± 0.041	n.d.	0.184 ± 0.016	0.175 ± 0.007 **	0.362 ± 0.052 *	n.d.	0.206 ± 0.016	0.281 ± 0.028	0.555 ± 0.077	n.d.	0.169 ± 0.004 *	0.195 ± 0.009 **	0.471 ± 0.073
Glycine	0.627 ± 0.070	0.215 ± 0.029	0.229 ± 0.015	0.187 ± 0.033	0.904 ± 0.144 *	0.257 ± 0.0075	0.228 ± 0.018	0.254 ± 0.021 *	0.871 ± 0.115 **	0.252 ± 0.009	0.178 ± 0.018 *	0.146 ± 0.026	0.744 ± 0.055	0.192 ± 0.010	0.153 ± 0.008 **	0.041 ± 0.072 *
Threonine	0.420 ± 0.007	0.649 ± 0.054	0.536 ± 0.022	0.315 ± 0.030	0.405 ± 0.022	0.668 ± 0.043	0.522 ± 0.047	0.475 ± 0.040 **	0.418 ± 0.033	0.642 ± 0.039	0.448 ± 0.045	0.262 ± 0.026	0.380 ± 0.048	0.577 ± 0.028	0.411 ± 0.026 **	0.390 ± 0.012 *
Citrulline	0.149 ± 0.024	0.166 ± 0.014	0.189 ± 0.025	0.103 ± 0.022	0.231 ± 0.032 *	0.238 ± 0.027 *	0.159 ± 0.014	0.059 ± 0.018	0.232 ± 0.013 **	0.218 ± 0.054	0.136 ± 0.005 *	0.066 ± 0.022	0.244 ± 0.009 **	0.281 ± 0.027 **	0.261 ± 0042	0.186 ± 0.027 *
Arginine	2.846 ± 0.430	4.421 ± 0.381	5.072 ± 0.281	4.43 ± 0.508	3.764 ± 0.234 *	4.386 ± 0.560	4.284 ± 0.217 *	3.649 ± 0.634	4.041 ± 0.174 *	4.808 ± 0.334	4.385 ± 0.338	3.481 ± 0.262 *	3.811 ± 0.141 *	4.967 ± 0.169	4.997 ± 0.170	5.241 ± 0.282
Taurine	0.024 ± 0.026	0.022 ± 0.008	0.020 ± 0.002	n.d.	0.021 ± 0.003	0.017 ± 0.002	0.013 ± 0.001 **	0.009 ± 0.001 **	0.010 ± 0.002	0.019 ± 0.002	0.011 ± 0.004 *	0.004 ±0.001 **	0.005 ± 0.001	0.012 ± 0.003	0.007 ± 0.006 *	0.033 ± 0.036
Alanine	0.561 ± 0.032	0.464 ± 0.028	0.192 ± 0.027	0.202 ± 0.020	0.663 ± 0.081	1.348 ± 0.156 **	1.086 ± 0.066 **	1.477 ± 0.090 **	0.574 ± 0.041	0.282 ± 0.014 **	0.148 ± 0.031	0.166 ± 0.010	0.393 ± 0.056	0.956 ± 0.024 **	0.489 ± 0.068 **	0.921 ± 0.107 **
Tyrosine	0.078 ± 0.008	0.378 ± 0.024	0.389 ± 0.027	0.350 ± 0.013	0.101 ± 0.010 *	0.611 ± 0.041 **	1.229 ± 0.136 **	3.270 ± 0.466 **	0.093 ± 0.005	0.321 0.025 *	0.348 ± 0.008	0.323 ± 0.018	0.100 ± 0.008 *	0.369 ± 0.030	0.604 ± 0.039 **	1.080 ± 0.139 **
Tryptophan	0.006 ± 0.009	0.083 ± 0.007	0.227 ± 0.013	0.490 ± 0.018	0.000 ± 0.001	0.065 ± 0.004 *	0.143 ± 0.010 **	0.338 ± 0.060 *	0.007 ± 0.003	0.077 ± 0.004	0.205 ± 0.020	0.511 ± 0.018	n.d.	0.055 ± 0.002 **	0.131 ± 0.004 **	0.396 ± 0.029 **
Valine	0.428 ± 0.022	1.011 ± 0.073	1.368 ± 0.065	1.431 ± 0.043	0.505 ± 0.023 *	1.090 ± 0.033	1.480 ± 0.104	2.044 ± 0.247 *	0.488 ± 0.110	1.016 ± 0.048	1.181 ± 0013 **	1.410 ± 0.063	0.431 ± 0.112	1.023 ± 0.027	1.338 ± 0.010	2.085 ± 0.139 **
Phenylalanine	0.032 ± 0.010	0.331 ± 0.017	0.791 ± 0.048	1.479 ± 0.045	0.026 ± 0.002	0.282 ± 0.019 *	0.350 ± 0.032 **	1.087 ± 0.142 *	0.027 ± 0.005	0.331 ± 0.022	0.729 ± 0.061	1.524 ± 0.160	0.017 ± 0.010	0.263 ± 0.003 **	0.533 ± 0.011 **	1.283 ± 0.071 *
Isoleucine	0.016 ± 0.010	0.305 ± 0.014	0.459 ± 0.046	0.507 ± 0.035	0.004 ± 0.002	0.349 ± 0.027	0.618 ± 0.049 *	0.989 ± 0.090 **	0.019 ± 0.008	0.300 ± 0.09	0.397 ± 0.004	0.458 ± 0.017	0.010 ± 0.001	0.306 ± 0.012	0.533 ± 0.022 *	0.953 ± 0.034 **
leucine	0.026 ± 0.012	0.291 ± 0.030	0.334 ± 0.039	0.395 ± 0.037	0.013 ± 0.003	0.423 ± 0.031 **	0.690 ± 0.078 **	0.923 ± 0.094 **	0.023 ± 0.004	0.267 ± 0.008	0.296 ± 0.021	0.382 ± 0.016	0.012 ± 0.001	0.341 ± 0.014 *	0.489 ± 0.030 **	0.742 ± 0.060 **
Ornithine	0.160 ± 0.025	0.310 ± 0.034	0.306 ± 0.038	0.178 ± 0.034	0.294 ± 0.010 **	0.777 ± 0.071 **	0.723 ± 0.075 **	0.434 ± 0.019 **	0.233 ± 0.032 *	0.291 ± 0.024	0.204 ± 0.020 *	0.123 ± 0.004 *	0.336 ± 0047 **	0.771 ± 0.009 **	0.762 ± 0.025 **	0.618 ± 0.131 **
Lysine	0.156 ± 0.015	0.606 ± 0.058	0.813 ± 0.050	0.555 ± 0.020	0.161 ± 0.006	0.597 ± 0.040	0.944 ± 0.058 *	1.380 ± 0.106 **	0.196 ± 0.018 *	0.590 ± 0.026	0.879 ± 0.054 *	0.417 ± 0.012 **	0.181 ± 0.004 *	0.533 ± 0.014 **	0.831 ± 0.008	1.174 ± 0.061 **

5. General Discussion and Conclusions

5.1. Alanine accumulation under low oxygen stress

Alanine accumulation is a well-known response of plants to low oxygen stress (Reggiani and Bertani, 2003). The current study (Chapter 2 and 3) was targeted to elucidate the mechanism of increased alanine synthesis under specific stress conditions and its physiological effects on the stress tolerance. AlaAT, which is induced by hypoxia, has traditionally been viewed as the enzyme responsible for the alanine production (Muench and Good, 1994). AlaAT was first investigated by characterizing the isolated *alaat1-1* mutant and the transgenic plants over-expressing barley *AlaAT* (Chapter 2). However, the results indicated that AlaAT was not required for the rapid accumulation of alanine under hypoxia, but was important for the conversion of alanine to pyruvate during the post-hypoxia. From this observation, another study was initiated to address the involvement of the GABA shunt in the alanine production under hypoxia (Chapter 3). Alanine can be synthesized in the second step of the GABA shunt, via the GABA-T reaction (Shelp *et al.* 1999). To investigate this possibility, GABA shunt deficient mutants (*gaba-t* and *gad* mutants) were isolated. Consequently, characterization of these mutants provided the evidence for alanine production through the GABA shunt; however, the GABA shunt pathway could not be fully accounted for all of the alanine synthesis under hypoxia as the rapid alanine accumulation was still observed in the GABA shunt deficient mutants. Since the alanine accumulation under hypoxia was not completely blocked in any of the mutants characterized in this study, the mechanism of alanine synthesis under the stress could not be fully elucidated. Furthermore, the physiological implications of alanine accumulation on the stress tolerance were not determined.

Nevertheless, this study revealed the complex nature of the amino acid metabolism under low oxygen stress. The results obtained here should be taken into consideration when designing future experiments studying plant amino acid metabolism under various stress conditions. In particular, it would be interesting to see if the GABA-shunt associated alanine synthesis could account for the frequently observed simultaneous accumulation of the two amino acids (alanine and GABA) under various stress conditions. In fact, the occurrence of both alanine and GABA at high levels in many previous experiments does not seem a coincidence (Sato *et al.*, 2002; Monselise *et al.*, 2003). Specifically, in some of the previous labeling experiments, the enrichment of [^{15}N] in GABA preceded that into alanine (Magalhaes *et al.*, 1990; Robinson *et al.*, 1991). These studies indicated that the GABA shunt contributes to substantial portions of alanine synthesis under certain conditions. Therefore, the operation of this pathway should be carefully monitored when the metabolism of alanine and GABA are studied in plants.

In terms of the mechanism of alanine production under hypoxia, the possibilities of other routes other than the AlaAT and GABA-T pathways and the simultaneous operation of multiple pathways are discussed here. Moreover, the discussion of the mechanisms in other distantly related organisms is also included in this section, since the increased alanine synthesis under hypoxia is a common phenomenon observed in most biological systems, including animals and some bacterial species (reviewed by Monselise *et al.*, 2003). In plants, other reactions leading to alanine production have been described in the past (Sousa and Sodek, 2003). Among these possible pathways are 1) transaminations involving pyruvate as the amino group acceptor, 2) alanine dehydrogenase (AlaDH), 3) aspartate β-decarboxylation.

The first pathway, alanine synthesis through transamination, involves the amino group transfer from amino acids to pyruvate to form alanine. This pathway seems to be the most plausible route for alanine production as previously suggested in plants (see Chapter 1) and as demonstrated in animals (by AlaAT) (Feala *et al.*, 2007). Besides, AlaAT and GABA-T, there have been reports of other aminotransferases in plants that accept other amino acids (e.g. glycine, serine, β-alanine) as amino group donors and pyruvate as the amino group acceptor (Liepman and Olsen, 2004). However, many of the older studies involved protein purification methods that were somewhat crude and our lab has observed these activities in earlier protein purification studies (Good, pers. comm.). These aminotransferases are also present in animals (*in silico* search, data not shown) and in bacteria (Oikawa, 2007). Additionally, there may be other unidentified aminotransferases capable of producing alanine in plants since many aminotransferase-like proteins are yet to be characterized (Liepman and Olsen, 2004). Since the alanine accumulation could not be blocked by the loss of either of AlaAT or GABA-T in this study, it is possible that alanine was produced by one of these aminotransferase or synthesized simultaneously through several of these enzymes. Considering the highly reversible nature of the transamination, the shift in the equilibrium of the reaction towards alanine synthesis may occur in response to hypoxia, which is known to increase the levels of pyruvate. Therefore, this shift may result in the alanine production through possibly multiple transamination routes. Also, it would be interesting to analyze the alanine accumulation in an *alaat1-1/gaba-t1-1* double mutant in a future study.

In the second potential pathway, alanine can be synthesized by AlaDH, which catalyzes the reductive amination of pyruvate to form alanine. The enzyme has been identified in several prokaryotes (Porum *et al.*, 1987; Schröder *et al.*, 2004), but has not been found in eukaryotes to date. Interestingly, over-expression of AlaDH has been used to produce alanine in *E. coli* (Lee *et al.*, 2004). The alanine production through AlaDH thus appears to be an efficient pathway to continuously produce this amino acid. In plants, previous attempts to detect AlaDH activity in plant extracts could not demonstrate the existence of this enzyme (Muench, pers. comm., Sousa and Sodek, 2003). Together with the failure of the current study to detect AlaDH activity in *Arabidopsis* extracts (data not shown), the occurrence of this enzyme in plants seems unlikely.

In the third pathway, alanine can be produced by the β-decarboxylation of aspartate. The possibility of the alanine synthesis from aspartate has received some attention in the past as the decrease in aspartate has been repeatedly observed under low oxygen stress in plants (Streeter and Thompson, 1972; Good and Muench, 1993). Conversion of aspartate to alanine reaction can be catalyzed by an enzyme called aspartate-β-decarboxylase (ASD). Although there have been only a few reports of the evidence supporting the presence of this enzyme in plants (Amagasa and Yoshida, 1981), the molecular identities of the enzyme have been known for bacterial enzymes (*in silico* search, data not shown). Interestingly, ASD is a protein evolutionally related to aspartate aminotransferase (AAT). Conversion of AAT into ASD by triple active-site mutations has been demonstrated in the *E. coli* protein (Braber *et al.*, 1999). Since several AAT genes, including eukaryotic- and prokaryotic-type enzymes, are present in plants (Torre *et al.*, 2006), it is not surprising that plants contain the functional enzyme. Nevertheless, the activity and the identity of ASD in plants have not been confirmed to date, and its potential involvement in the alanine accumulation under hypoxia remains unclear. In contrast, bacterial ASD was identified long ago and has been used to commercially produce alanine in bacteria (Shibatani *et al.*, 1979). Since the reaction is irreversible like GAD, alanine production appears to continue as long as aspartate is sufficiently supplied.

In order to give insights into the precise routes for alanine production, additional experiments were performed to analyze the effects of the exogenous supply of glutamate or aspartate, and the addition of the GS inhibitor, MSX, on the alanine accumulation under hypoxia (Figure 5.1). In this test, both glutamate and aspartate addition increased the alanine accumulation under hypoxia (Figure 5.1A). The results suggest that these amino acids are used for the synthesis of alanine. However, which amino acids (Glu, Asp or other amino acids derived from them) donate their amino group to pyruvate could not be determined in this experiment. Also, whether the alanine synthesis under hypoxia requires the ammonium assimilation into glutamine was investigated by blocking GS with MSX. In the presence of MSX, significant inhibition of the hypoxia-induced alanine accumulation was observed (Figure 5.1B). Thus, the results clearly demonstrated that the large portion of N deposited into alanine during hypoxia was derived from ammonia assimilated into glutamine by GS. The experiment conducted here was similar to that performed by Reggiani *et al.* (2000), and both studies resulted in comparable results. From these observations, the amino group of alanine appears to derive from other amino acids. This supports transaminations as the major pathways of alanine synthesis; however whether the alanine synthesis occurs through single transamination or through multiple transaminations needs further investigations. To identify the precise route of the amino group transfer to pyruvate, ^{15}N-labeling experiments with only short time intervals (e.g. every 10 min) would be required. The use of an NMR analysis would also be beneficial in this type of study as it allows the non-destructive monitoring of the movement of ^{15}N label in a continuous time-scale.

Although the physiological importance of the alanine production on hypoxic stress tolerance was not demonstrated, the role of the transamination-dependent alanine synthesis may lie in the energy conservation during the stress conditions. In rice, the importance of decreasing the rate of carbohydrate consumption during submergence was recently demonstrated by a study addressing the role of *Sub1A* (Fukao et al., 2006). Since the glycolysis pathway requires NAD^+ as an oxidant, the important role of ethanol and lactate fermentation pathways is to regenerate NAD^+ from NADH. In contrast, alanine synthesis does not produce NAD^+, but it functions to prevent the accumulation of the end product of the glycolysis. In this sense, alanine production may not be a favorable reaction as it could reduce the NAD^+/NADH ratio, resulting in a decrease in metabolic flux through the glycolytic pathway. However, it could be viewed that the role of the alanine synthesis under hypoxia is to facilitate this decrease in the carbohydrate catabolism, since the energy conservation has been recently suggested as a major controlling factor for the survival of plants under low oxygen stress (Fukao et al., 2006). If this is the case, alanine production during low oxygen stress seems to be an efficient energy conservation mechanism as the produced alanine can be re-used as respiration substrates upon return to normoxia. Nonetheless, this needs to be experimentally demonstrated, possibly by analyzing mutants that do not accumulate alanine under hypoxia.

In summary, the results of the current study indicated that alanine can be synthesized in pathways other than AlaAT since the *alaat1-1* and the *alaat1-1/alaat2-1* double mutant accumulated alanine under hypoxia (Chapter 2). These results raised a question about the role of AlaAT that was previously believed to be the major alanine-producing enzyme. As the potential alternative route for the alanine synthesis, the GABA shunt pathway was also studied (Chapter 3). The results demonstrated that some of alanine production could be attributed to this pathway. Since the GABA-TP (GABA;pyruvate transaminase) is unique to plants, this unidirectional alternative route may account for the exceptionally high levels of the alanine accumulation in plants compared to other organisms.

5.3. Role of glutamate dehydrogenase in plants

GDH had been viewed as the major ammonium assimilatory enzyme until GS/GOGAT pathway was demonstrated to be the port of ammonium assimilation (Lea and Miflin, 1974). Since then, the role played by GDH has been a heated topic debated over several decades by plant physiologists (Miflin and Habash, 2002; Dubois *et al.*, 2003). The controversy of plant GDH was mainly confounded by the uncertainty regarding the *in vivo* direction of the enzyme reaction. Many labeling experiments have repeatedly indicated that the oxidative deamination is the *in vivo* direction of the reaction (Dubois *et al.*, 2003). In two recent studies, either the α or β subunit of GDH was over-expressed in transgenic plants (Purnel and Botella 2007; Skopelitis *et al.*, 2007). These transgenics contained the increased activity of isoenzyme 1 (β_6) or isoenzyme 7 (α_6) due to the expression of the transgene. Labeling experiments using these transgenic plants revealed the increased release of $^{15}NH_4^+$ after [^{15}N]glutamate feeding, demonstrating the deaminating role of the two GDH homohexamers (Purnel and Botella 2007; Skopelitis *et al.*, 2007). These results provided the additional evidence supporting the current consensus that GDH deaminates glutamate *in vivo*. Nonetheless, a contrasting result was obtained from the experiment conducted under salt stress where reductive amination of 2-oxoglutarate by GDH was demonstrated as $^{15}NH_4^+$ incorporation occurred even in the presence of the GS inhibitor, MSX (Skopelitis *et al.*, 2006). Taken together, it appears that GDH deaminates glutamate under normal growth conditions but may catalyze the reductive amination only under certain stress conditions (e.g. salt stress). However, evidence supporting the latter aminating role of GDH is still scarce. In the current study, the phenotype observed in the *gdh* mutants supported only the deaminating role of GDH under the conditions tested, although the direction of the enzyme reaction was not tested by labeling experiments.

Although labeling experiments were useful to define the *in vivo* direction of the reaction, the physiological role of GDH remained speculative. For this reason, GDH expression has been extensively studied at the transcript, protein and enzyme activity levels in several plant species. In these previous studies, the regulation of GDH by light/dark cycle, sugar levels and ammonia addition was documented (Dubois et al, 2003). In brief, expression of *GDH* appears to be controlled by the cellular energy state or sugar levels since the dark-induction of *GDH* can be repressed by exogenous addition of sucrose (Melo-Oliveria *et al.*, 1996; Purnel *et al.*, 2005). This tight regulation of GDH according to the cellular C status has led researchers to suggest that the deamination of glutamate by GDH is important to supply alternative C source to the respiratory pathway during sugar starvation (Melo-Oliveira *et al.*, 1996; Dubois *et al.*, 2003). However, this hypothesis has not been demonstrated until recently, since the previously characterized *gdh* mutants as well as the transgenic plants over-expressing either of *GDH* genes did not exhibit a visible phenotype that supports the proposed role of GDH (Magalhaes *et al*., 1990; Melo-Oliveira *et al*., 1996; Fontaine *et al*., 2006; Purnel and Botella, 2007; Skepelitis *et al*., 2007). All previous *gdh* mutants contained mutations only in one of the two *GDH* genes. It is possible that the effects of the loss of one *GDH* were masked by the redundant function of the other *GDH* in previous studies. In this thesis research (Chapter 4), I have isolated mutants for both of the two known *Arabidopsis GDH* genes and obtained a *gdh* double mutant (*gdh1-2/gdh2-1*) from crossing *gdh1* and *gdh2* mutants. Using these mutants, I studied the role of GDH during sugar starvation by subjecting the plants to prolonged darkness. This experiment revealed the increased susceptibility of *gdh1-2/gdh2-1* to prolonged darkness, the first visible phenotype associated with the loss of GDH activity. The recovery of the phenotype of *gdh1-2/gdh2-1* by the addition of sucrose before the onset of the dark treatment indicated that dark-induced C starvation was the cause of the conditional phenotype. In addition, the functional redundancy of the two *GDH* genes was supported by the absence of the phenotype in both of the single mutants. Based on the view that GDH deaminates glutamate *in vivo*, the anaplerotic GDH reaction functions to funnel the C skeleton of glutamate into the TCA cycle during sugar starvation.

Quantification of amino acid levels in the isolated *gdh* mutants also revealed the profound effects of the loss of GDH activity on amino acid metabolism during C limitation (Chapter 4). In general, sugar starvation accelerates the protein degradation through the autophagic process, resulting in the increase in free amino acids (Yu, 1999). Under such conditions, catabolism of the accumulated free amino acids becomes important as the source of respiratory substrates. In the current study (Chapter 4), levels of several amino acids differed significantly between WT and the *gdh* mutants during the dark treatment, although the amino acid profile of WT and those of the *gdh* mutants were similar under normal conditions. During the dark treatment, levels of alanine and aspartate decreased in WT, but conversely, increased in the *gdh* mutants. Also, an increased accumulation of branched-chain amino acids (i.e. leucine, isoleuccine and valine) was observed in *gdh1-2/gdh2-1*. Interestingly, increase and decrease in glutamate levels were observed in WT and *gdh1-2/gdh2-1*, respectively. Based on these observations, I propose that glutamate deamination by GDH is essential for generation of 2-oxoglutate, which acts as an amino group acceptor in the transaminations required for the breakdown of several amino acids (Figure 5.2). The amino acid species over-accumulated in *gdh1-2/gdh2-1* during the dark treatment were in fact those that can be converted to respirable 2-oxoacids through the transaminations. Therefore, glutamate deamination by GDH appears to play a central role in amino acids catabolism. Co-localization of GDH and various aminotransferases in the mitochondria (Liepman and Olsen, 2004) further supports the proposed model.

In order to respire amino acids, all amino acids must be deaminated before they are fed into the TCA cycle. The direct release of ammonia from amino acids can be catalyzed by specific enzymes for only a handful of amino acid species in plants (i.e. GDH, glycine decarboxylase, threonine deaminase, phenylalanine lyase, arginase, asparaginase, glutaminase). It should be noted that amino acid oxidase, which hydrolyzes broad amino acid species in an oxygen-requiring reaction that produces H_2O_2, is not known in plants. In the proposed model of amino acid catabolism, the amino groups of several amino acids are first transferred to the amino N of glutamate by aminotransferases. GDH then catalyzes the deamination of glutamate, completing the ammonia release form amino acids. Interestingly, this model is analogous to the well-characterized amino acid catabolism in animals where GDH plays a similar key role in the deamination of amino acids (Brosnan, 2000). To avoid the toxic buildup of ammonia in the cell, released ammonia seem to be re-assimilated into glutamine by GS in plants (Figure 5.2), while ammonia enters urea cycle in mammals (Brosnan, 2000). In future studies, [^{15}N] amino acids (e.g. glutamate, alanine, aspartate, branched-chain amino acids) feeding can be used to see if the release of $^{15}NH_4^+$ is affected in the *gdh* mutants. Decrease in $^{15}NH_4^+$ release in the *gdh* mutants in the future experiment would further demonstrate the involvement of GDH in the ammonia release from several amino acid species.

The proposed role of GDH in amino acid catabolism also indicates the potential involvement of GDH in N remobilization during senescence. In fact, induction of GDH in senescing leaves was documented in plants (Masclaux *et al.*, 2000; Masclaux-Daubresse *et al.*, 2006). Since the dark-induced C starvation mimics the physiological process of senescence, the deamination catalyzed by GDH appears to be an important source of ammonia that is re-assimilated into N transport metabolites (mostly glutamine and asparagine). This potential role of GDH during senescence is particularly noteworthy since cytosolic GS1 has been recently shown to be the major component that determines the grain production in maze (Martin *et al.*, 2006). The co-induction of cytosolic GS1 and GDH during senescence (Masclaux *et al.*, 2000; Masclaux-Daubresse *et al.*, 2006) points to an efficient pathway connecting the synthesis of glutamine with the degradation of amino acids (Figure 5.1). In the support for this model, transaminations may become the important route of glutamate formation in senescing leaves as GOGAT activity generally decreases in older leaves (Masclaux *et al.*, 2000). In fact, the current study showed the glutamate increase in WT but a contrasting decrease in the *gdh* mutants, during dark treatment (Chapter 4); consistent with the hypothesis that glutamate is formed by transaminations during dark-induced C starvation. A labeling study monitoring $^{15}NH_4^+$ incorporation in the presence or absence of GOGAT inhibitor, AZA, also demonstrated that ammonium assimilation into glutamine (and to a lesser extent, into asparagine) is not affected by the inhibition of GOGAT in the dark, indicating that glutamate can be produced by reactions other than GOGAT (Masclaux-Daubresse *et al.*, 2006). These data suggest that the amino acid catabolism and the synthesis of N transport compounds (glutamine and asparagine) can proceed simultaneously without GOGAT activity, in the dark or during senescence. In rice, glutamine formed in senescing leaves is translocated to sink organs and then converted to glutamate therein by the GOGAT expressed in the recipient cells (Tabuchi *et al.*, 2007). The sequential events during the senescence process, 1) protein degradation, 2) amino acid breakdown, 3) synthesis of N transport compounds (glutamine and asparagine) allows the efficient remobilization and the translocation of proteinous N from source leaves to sink organs. Whether the glutamate deamination catalyzed by GDH plays an important role in the remobilization of stored N in old leaves is an interesting topic for the future study.

In conclusion, the current study, which isolated and characterized the *gdh* mutants, gave insights into the physiological role of GDH in plants. Results indicated that GDH is involved in amino acid catabolism in plants. However, this study has only laid the groundwork for the characterization of GDH in plants, and further investigations are necessary to more precisely define the role of this long-disputed enzyme.

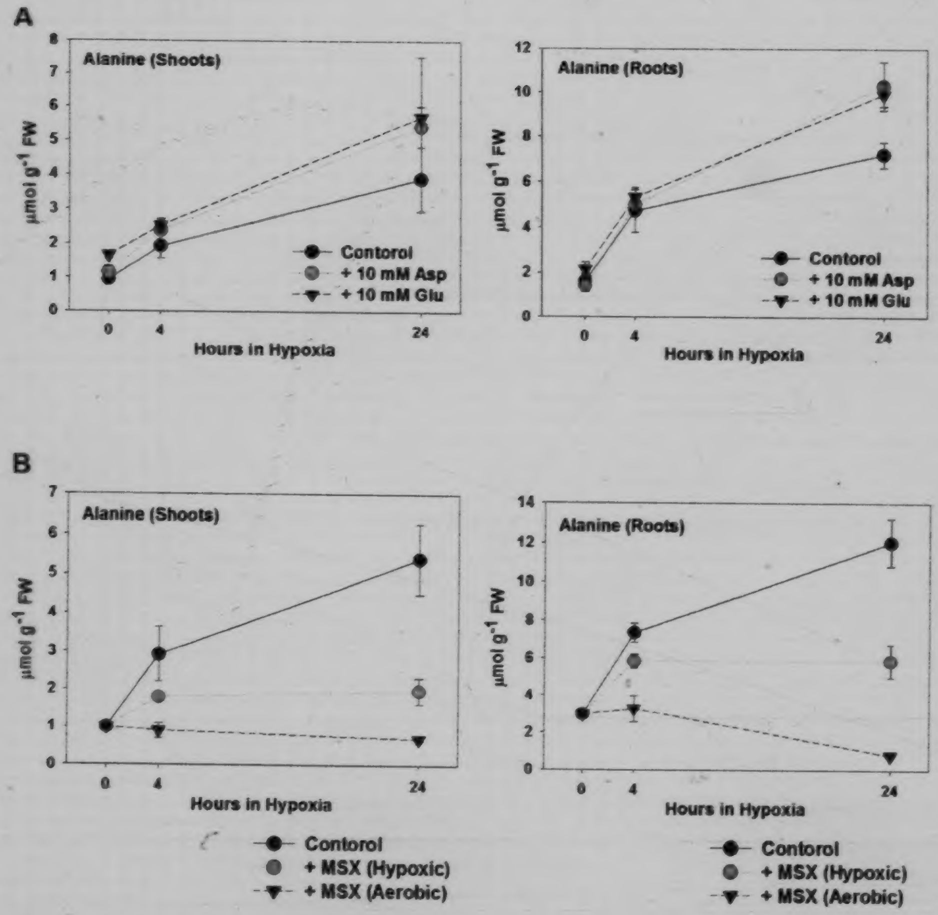

Figure 5.1. Effects of exogenous supply of aspartate and glutamate as well as the addition of MSX on the alanine accumulation under hypoxia.

A. Aspartate or glutamate was added to the liquid 0.5x MS media to the final concentration of 10 mM before subjecting the plants to the hypoxic treatment (5% O_2). B. The GS inhibitor, methionine sulfoximine (MSX), was added to the liquid 0.5x MS media to the final concentration of 1 mM, 2 h before the hypoxic treatment. As the control experiments, MSX was not added to the subsets of the plates. Also, the subsets of the plates that received the MSX addition was not treated for hypoxia. The hypoxic treatments were performed as described in Chapter 2. The 17-day-old WT (Col-0) *Arabidopsis* plants grown as described in Chapter 2 were used for all experiments. The error bars indicate S.D.

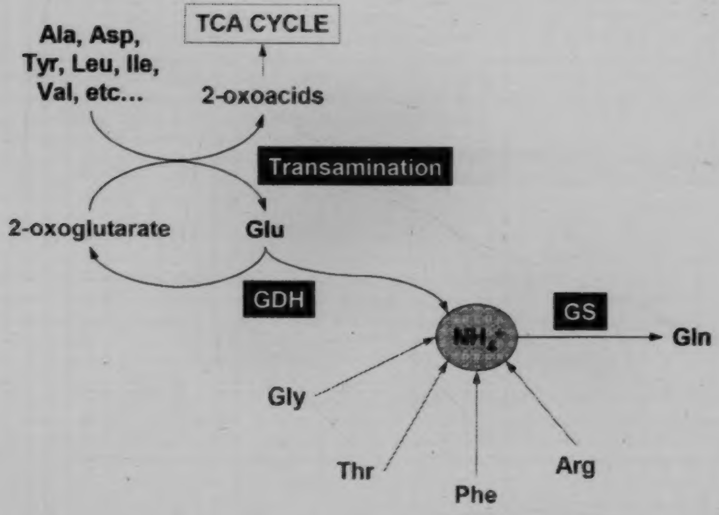
Ala, Asp,
Tyr, Leu, Ile,
Val, etc...
TCA CYCLE
2-oxoacids
Transamination
2-oxoglutarate
Glu
GDH
NH4+
GS
Gln
Gly
Thr
Phe
Arg

Figure 5.2. Proposed model of amino acid catabolism during sugar starvation.

Deamination of glutamate is catalyzed by GDH. The enzymes such as glycine decarboxylase, threonine deaminase, phenylalanine lyase and arginase also catalyze the deamination of the corresponding amino acids. Among these deaminating reactions, GDH appears to be the major route for amino acid breakdown as the amino group of other amino acids can be transferred to Glu through transaminations. The released ammonia can be re-assimilated into Gln by glutamine synthetase (GS). During senescence, Gln can be translocated to sink tissues.

5.3. Bibliography

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